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### *Self-incompatibility in ryegrass: Homing in on the genes of the S-locus complex*

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# Proceedings of the 7th International Symposium on the Molecular Breeding of Forage and Turf

MBFT2012 – Salt Lake City, Utah, USA

**June 4-7, 2012**



# Proceedings of the 7th International Symposium on the Molecular Breeding of Forage and Turf

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The seventh International Symposium on the Molecular Breeding of Forage and Turf, MBFT2012, was held in Salt Lake City, UT, USA, from June 4 – 7, 2012. One-hundred and fifteen researchers from around the world presented oral and poster formats relating to ten general topics: Genetic mechanisms and applications, comparative genomics, herbage quality, symbionts, bioenergy, germplasm/diversity/and its impact on breeding, abiotic and biotic stresses, genomic selection and plant improvement, functional genomics and gene discovery, and transgenic processes and procedures. A tour was included to forage research plots at Evan's farm, National Turfgrass Evaluation Program and other turf research plots at Greenville farm, and grazing research at Lewiston farm; all used by the USDA-ARS Forage and Range Research Laboratory and Utah State University. In this proceedings are selected manuscripts of invited speakers, and abstracts of oral and poster presentations. We thank the participants and organizing committee for the outstanding research and presentations at this symposium.

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# The Current Status of Metabolomics and Its Potential Contribution to Forage Genetics and Breeding

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## Current status of metabolomics

Metabolomics provides a systems-level measurement for the collection of all metabolites; the end products of cellular processes. Metabolomics together with genomics, transcriptomics, and proteomics are powerful technical platforms which help understand molecular pathways from genomic constituents to the expression of the final trait. Unlike the well-structured large DNA and protein molecules, metabolites vary enormously in their structure and physicochemical properties. This makes the high throughput characterization of the complete set of metabolites in an organism a daunting task.

Mass spectrometry, either direct infusion-based or coupled with chromatography, has become the dominant technical platform in metabolomics due to its high sensitivity, high sample throughput, accurate detection of mass-over-charge ratio ( $m/z$ ), and compact instrumentation. Soft ionization methods such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) allow direct analysis of polar and thermally labile biomolecules in their intact form. Thanks to the advances in high-resolution mass spectrometry and high-resolution chromatography thousands of peaks (described as  $m/z$  and retention time) can be routinely identified from crude extracts of biological samples. However, a few issues must be addressed for reliable quantification of peaks and the identification of metabolites from peaks. Systematic variations in metabolomic studies have been documented including retention time shifts, variations of peak quantification between batches and run-order effects due to decreased ionization efficiency within a batch. The use of known standards to quantify all of the detected entities becomes impractical in metabolomics. Computational tools are employed to achieve relative quantifications among biological treatments.

Structural elucidation of all the genuine peak signals remains an extremely challenging task (Kind and Fiehn 2010). High accuracy  $m/z$  measurement allows the prediction of elemental formula of identified peaks but is limited to those peaks of very low molecular weight. ESI MS/MS fragmentation spectra are routinely used for peptide sequencing and putative structural interpretation of small molecules, however, the understanding and interpretation of ESI fragmentation pathways remains challenging for small molecules due to their diverse structures and the sparse fragment spectra. Peak annotation is complicated by (de)protonation, metal ion adducts, isotopic ions, charge states and in-source fragments. Databases based on spectral features like accurate  $m/z$ , retention time, ion fragmentation patterns and other information are being developed to aid metabolite identification. MassBank ([www.massbank.jp](http://www.massbank.jp)) is such an example of spectral data repository from plant species

Despite the current challenges, metabolomics has proven a valuable tool in correlating biochemical variations with phenotypes. A common practice is to identify biologically significant signals first via computational and statistical tools, and then structural interpretations can be carried out for the top-ranked signals. Unknown or unexpected metabolites are usually found for a characteristic of biological relevance, which indicates the extent to which our picture of cellular metabolism is incomplete (Patti et al. 2012). Over the years several analytical platforms have been developed at AgResearch and applied to the identification of new metabolites involved in the ryegrass and endophyte associations (Cao et al. 2008; Koulman et al. 2012); the formation of testable hypotheses from co-regulation of gene expression and metabolite changes (Cao et al. 2007); and the fingerprinting of ryegrass and white clover germplasm. A recent review (Rasmussen et al. 2012) summarized the broad aspects of recent advances on forage metabolic responses to osmotic stress, nutrients, and fungal associations.

## Application of metabolomics to forage genetics and breeding

The majority of traits that are of interest to forage breeders, such as yield, digestibility, and tolerance to biotic/abiotic stresses are complex characteristics. Selection for these traits based on genetic markers alone has proven challenging (Collard and Mackill 2008). The reasons for this are manifold and may include polygenic nature, complexity of G×E and over-simplified description of phenotypes.

With the increasing capacity of genotyping the characterization of phenotypes often remains superficial and the main bottleneck to many genetic studies (Myles et al. 2009). Evaluation of phenotypes on collected germplasm is necessary for designing a breeding program, but it is often not straightforward and the cost is high. Metabolomics enables detailed understanding of biochemical regulation of complex traits such as drought response (Oliver et al. 2011), characterization of the phenotype of silent mutations (Raamsdonk et al. 2001) and classification of germplasm or breeding lines (Fig. 1). A phenotype dissected at the metabolic level may allow more precise evaluation of forage nutritive quality than those of traditionally used criteria such as crude protein (CP), water-soluble carbohydrate (WSC) and crude fibre (NDF). On the other hand, metabolites may form the end phenotype that could be subject to direct selection. For example, defence compounds could be selected for disease resistance rather than using subjective disease resistance scores. The application of metabolomics to genetic populations has many opportunities, including the identification of genetic loci conferring the expression of significant metabolites (Koulman et al. 2009) and the identification and cloning of novel genes associated with a metabolic disruption demonstrating that metabolic phenotypes can provide mechanistic insights into gene function (Dumas et al. 2007).

Forage plants have been considered recalcitrant to genomic studies because of their high genetic heterozygosity. The decreasing cost of genotyping, however, has enabled high capacity genomic methods to come within the reach of these species. We anticipate that high throughput next-generation sequencing, metabolomics and bioinformatics will provide new avenues for rapidly advancing our understanding of the genetic regulation of natural products and metabolic processes that are predictive of complex traits (Riedelsheimer et al. 2012). Genetic mapping of metabolic variation, be it either family or population-based, has revealed novel associations between gene function and metabolism. As an example, a single genetic locus was reported to be associated with the production of a number of related bioactive molecules, suggesting a promising opportunity for molecular breeding of bioactive metabolites (Winzer et al. 2012).

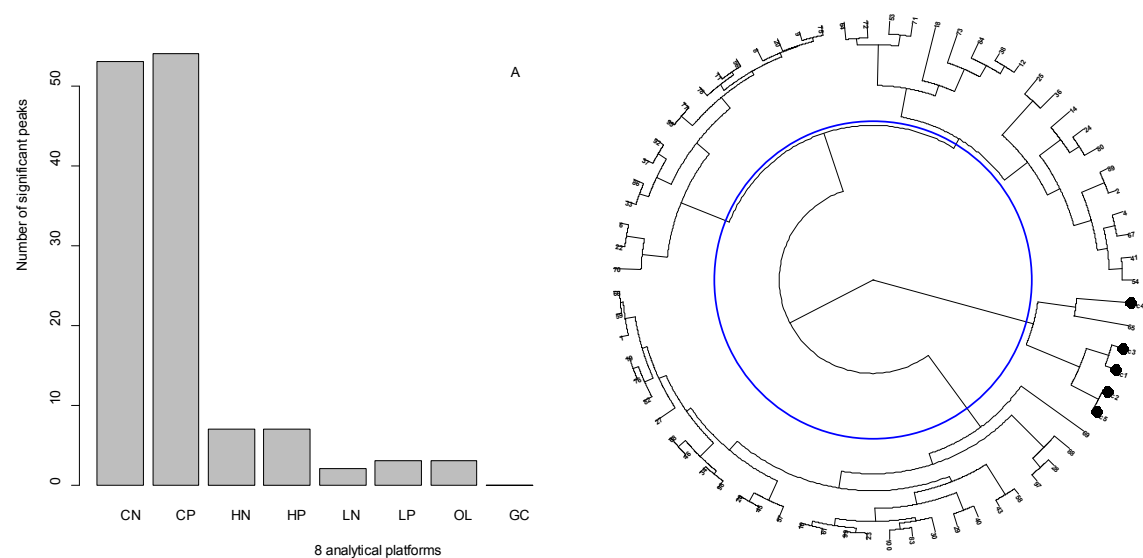


Figure 1. (A) Metabolomics based on 8 analytical platforms (\*) was applied to investigate metabolic contents of a full sib backcross family ( $BC_1$ ) derived from white clover and *T. uniflorum*. From 129 differentially expressed peaks it is indicated that different metabolites between white clover and the  $BC_1$  population are secondary metabolites (rather than primary metabolites such as carbohydrates and lipids). (B) White clover cultivars (5 emboldened termini) cluster with similar metabolic signatures. Based on the same criteria another three clusters occurred among the  $BC_1$  individuals.

(\*) the 8 analytical platforms include C18 reverse phase LCMS in positive (CP) and negative (CN) ionization mode, hydrophilic interaction LCMS in positive (HP) and negative (HN) mode, LCMS for the analysis of lipids (LP, LN) and oligosaccharides (OL), and GCMS (GC) for volatile compounds.

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# Marker-assisted selection using QTL-linked SSR markers in temperate forages

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## Introduction

Profitable production from pastoral agriculture in New Zealand is reliant on persistent pastures, which are based primarily on perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.). Breeding has, historically, delivered modest genetic gains in these species - estimated at 0.2 – 0.6% per annum (pa) for herbage yield in perennial ryegrass (Van Wijk and Reheul 1991; Easton et al. 2002; Sampoux et al. 2010) and 0.6 – 1.3% pa for white clover (Woodfield & Caradus 1994; Woodfield 1999). Higher rates of genetic gain in forages are needed to enable improved pasture performance in the face of challenges, including agricultural intensification, increasing ruminant genetic potential and abiotic and biotic stresses.

Marker-assisted selection (MAS) may enhance genetic gain in breeding programmes by using molecular markers to efficiently and precisely select plants with desirable gene variants for traits of interest. Numerous quantitative trait loci (QTL) have been identified for agronomically important traits in forage legumes and grasses but, due in part to the obligately-outcrossing, heterozygous nature of these species, there are few reported examples where markers linked to these QTL have been implemented in MAS (Humphreys and Turner 2001; Dolstra et al. 2007; Stendal et al. 2006).

Barrett et al. (2008) used markers associated with seed yield QTL in white clover to screen breeding populations and successfully identify marker alleles that had a significant effect on seed yield in two-thirds of populations surveyed. Here we have tested a similar approach for complex traits in perennial ryegrass (herbage yield, HY) and white clover (average node number, ANN). Microsatellite (SSR)-based genetic linkage maps developed previously were used to identify QTL for herbage yield-related traits in ryegrass (Sartie et al. 2011; Faville et al. 2012) and ANN in white clover. Markers linked to QTL regions identified in these biparental populations were screened for detection of marker:trait associations in multi-parent breeding populations and then applied in selection cycles to support proof-of-concept evaluation of MAS efficacy in forages.

## Ryegrass

Individual plants from two perennial ryegrass breeding populations (GA207 and GA208) were evaluated seasonally for HY, measured both as vigour score and dry matter (DM) yield, in a mixed species sward, over two years at Palmerston North, New Zealand. Twelve significant ( $P < 0.001$ ) marker:trait associations, determined by regression of marker data (allele or genotype) on best linear unbiased predictors (BLUPs) for HY measures, were detected using HY QTL-linked SSRs. Plants lacking a marker allele at one locus on linkage group (LG) 6 exhibited mean 23% (population GA208) and 11% (GA207) increases in annual DM yield above the sub-populations that did not carry the allele.

Phenotypically equivalent, marker-divergent selections based on a single marker locus (M+, mean DM of 12.8 g  $\pm$  1.10 SD; M-, mean DM of 12.2 g  $\pm$  0.61) were made within population GA208 and polycrosses completed within each selection class. Balanced bulks of half-sib (HS) progeny seed from M+ (lacks detrimental allele) and M- (contains detrimental allele) selections were assessed in replicated single row plots at Palmerston North. Performance in rows to date shows evidence of divergent HY means consistent with observed marker effects in the parents, with M+ exceeding DM yield of M- by up to 29% between November 2011 and March 2012. Seed increases from M+ and M- are being completed to enable a more comprehensive, multi-environment evaluation of marker effects on HY (and correlated traits) to verify efficacy of this approach to MAS in perennial ryegrass.

## White clover

In white clover, the persistence related traits ANN and internode length (IL) were selected. For each trait, six QTL-linked SSRs discovered in mixed-sward field trials (multi-year and multi-site) were used to screen a multi-parent population sampled from an early generation of the cultivar 'Kopu II'. Significant ( $P < 0.0001$ ) marker:trait associations were identified for each trait. Five SSR alleles with significant marker:trait associations were used to develop divergent marker selection indices, for increased or reduced ANN. For ANN, 'Kopu II' population plants carrying the beneficial marker index exhibited 19% higher mean ANN than those without.

Contrasting selections (M+ and M-) were made from within the 'Kopu II' population on the basis of both phenotypic and marker-based selection indices, and polycrosses completed within each selection class. HS progeny (15 families × 10 plants each) from M+ and M- selections were evaluated for ANN in pots. The M+ and M- HS progenies exhibited a 19% difference ( $P < 0.05$ ) in mean trait performance, the same level observed in the parental generation, indicating that MAS was effective. These same HS progeny are now being tested in mixed sward field environments.

## Conclusions

These cases describe a pragmatic approach to QTL-targeted MAS and demonstrate the potential for detecting substantial, single marker effects in the context of an applied breeding programme for outbreeding forage plant species. Markers from biparental QTL detected associations with target traits in genetically-complex breeding populations and predicted the performance of half-sibling progeny from MAS. Although there are caveats (general population specificity of allele:trait association; dependence on population linkage disequilibrium levels; correlated effects on important traits); these findings validate the efficacy of MAS in obligate outcrossing forages using established marker systems and breeding structures, delivering substantial differences on economic traits on the basis of single marker assays.

## Acknowledgements

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# A search for candidate genes affecting late heading in orchardgrass/ cocksfoot (*Dactylis glomerata* L.)

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## Introduction

Orchardgrass (*Dactylis glomerata* L.) is indigenous to Eurasia and northern Africa, but has been naturalized on nearly every continent. Despite its distribution and uses, there is a need for improved late flowering germplasm for use in North American mixed pastures. Many candidate genes affecting heading date exist in cereal grasses, including vernalization (*Vrn*) (Dubcovsky et al. 2005; Karsai et al. 2008), flowering time (*Ft*) (Skøt et al. 2011), and heading date (*Hd*) genes (Nemoto et al. 2003; Skøt et al. 2007). An F<sub>1</sub> population derived from a very late heading *D. glomerata* ssp. *himalayensis* parent and an early to mid-heading *D. glomerata* ssp. *aschersoniana* parent was previously published (Xie et al., 2012), wherein heading dates were measured over two years and individuals were genotyped using a combination of EST-derived SSR markers and AFLP markers. Six of the seven orchardgrass linkage groups were assigned based on this putative synteny with rice, and QTL were detected for heading date on linkage groups 2, 5, and 6 in both parental maps. This paper describes an effort to map the three candidate genes listed above in this mapping population, to determine if any of them coincide with heading date QTLs.

## Orchardgrass EST database search

DNA sequence of the candidate genes was first searched using the *Dactylis glomerata* EST database (<http://titan.biotech.uiuc.edu/dactylis/>) (Bushman et al., 2011). The ESTIMA database annotations are updated semi-annually. Using keyword searches, no vernalization, heading date, or flowering time genes were found. Using sequences from perennial ryegrass (*Lolium perenne* L.), and BLASTn algorithms with E-values of 100, no sequences were identified.

## Solution Capture Sequencing

In order to obtain sequences of candidate genes, solution capture sequencing was employed with the MYselect kit (MYcroarray, Ann Arbor, MI, USA) on genomic DNA from the *D. glomerata* ssp. *himalayensis* and ssp. *aschersoniana* parents of the F<sub>1</sub> mapping population reported in Xie et al. (2012). Solution capture, or targeted enrichment, has the benefit of enriching genomic sequences that contain introns and untranslated regions. For this experiment we used synthetic oligos designed from expressed or heterologous sequences. Sequences of candidate genes from perennial ryegrass, tall fescue (*Festuca arundinaceae* Schreb), and barley (*Hordeum vulgare* L.) were aligned to each other and used for orchardgrass bait design. For the *Vrn* genes, Genbank accession numbers EU007834, FJ793194, FJ687750, and GQ227989 were used to design baits (Table 1). For the *Hd* genes, accession numbers AB094488, AB094481, and AM489608 were used. For the *Ft* gene(s), FN993928 and FN993915 were used. At least two bait regions were designed from each candidate gene to encourage more gene coverage. Manual selection from heterologous bait sequence regions was required to avoid selecting bait regions that included intron/exon junctions, which would not bind to genomic DNA in downstream steps. Because too few bait sequences preclude successful targeted enrichment, extra orchardgrass sequences from the ESTIMA EST library were also included as baits, resulting in 77 bait sequences for oligo design. Solution capture followed the manufacturer's recommended protocol, with one exception. Due to the low yield from the fast library preparation, 10 PCR reactions for each library were combined and concentrated to increase the concentration of every library before moving on to the solution capture.

Table 1. Sequences used to create baits for three flowering time genes, the targeted enrichment contig identifier, and the portions of the bait sequences with hits to the orchardgrass sequences.

Gene	Bait Gene	Genbank Accession of Bait Gene	Hit ID	Hit Region (bp of Bait Gene)	Note
Hd	TaHd1-2	AB094488	Contig2	27-123	5'UTR
	TaHd1-2	AB094488	Contig558	1742-2008	exon2
	Tahd1-3	AB094491	Contig558	1077-1310	exon2
	LpHd1	AM489608	Contig981	1950-2378	exon2 and 3'UTR
Ft	LpFT3	FN993928	Contig404	1120-1397 /1423-1505/1603-1738	exon3 and 3'UTR
	FeFT3	FN993915	Contig404	1419-1503/1598-1701	3'UTR
Vrn	FeVRN1	FJ793194	Contig441	148-336	exon1
	LpVRN1	GQ227990	Contig441	638-826	exon1
	VRN1	FJ687750	Contig441	1999-2189	exon1
	HvVRN-H3/FT1	EU007834	Contig404	800-1066	exon3

Captured products were used as templates for Emulsion PCR and pooled into a ¼ 454-Roche sequencing chip at the Utah State University Center for Integrated Biosystems (CIB). Sequencing data were analyzed using CLC Genomics Workbench (CLC bio, Cambridge, MA, USA). Approximately 21,000 reads were generated from 454 sequencing, which assembled into 2,094 contigs varying in size from 105 bp to 1,975 bp. After BLASTn searches, 106 contigs had hits to 64 (83%) of the 77 bait sequences; including the *Vrn*, *Hd*, and *Ft* candidate genes (Table 1). Although bait sequences were homologous with all three *Vrn* genes, only contigs with hits to *Vrn1* and *Vrn3* genes were found (Table 1). The Contig441 hit *Vrn1* exon1 sequences from the bait accession numbers, and Contig 404 hit exon3 of the *Vrn3* gene. For the *Hd* gene family, bait sequences were designed from *Hd1*, and three contigs had hits to a region upstream of the start codon as well as the second exon of this gene (Table 1). For the *Ft3* gene, which is synonymous with *Vrn3*, Contig 404 had hits to the last exon and 3'UTR portions of the gene.



## Genetic Mapping of *Vrn3/Ft3*

Of these contigs, 18 candidate gene primer pairs were designed and tested for likely suitable segregation ratios in the orchardgrass  $F_1$  mapping population. Five primer pairs showed polymorphism in a test panel of 10 plants, and *Vrn3* was successfully mapped at a 1:1 segregation ratio on linkage group 3 of *Him271* genetic map (Figure 1).

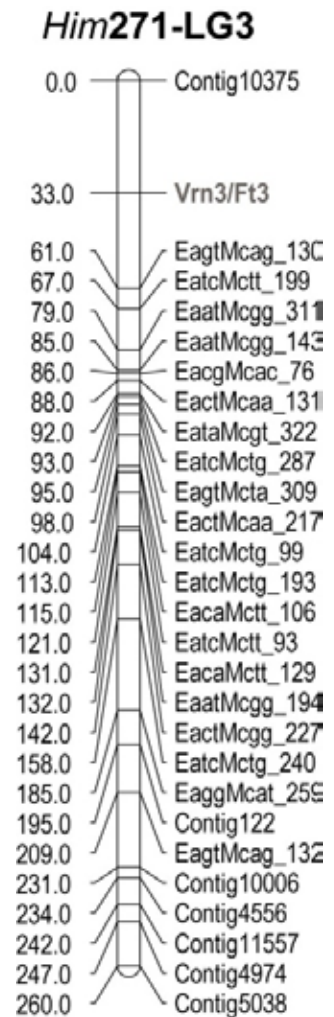
## Conclusion

Heading date is the primary factor determining the switch from vegetative to reproductive phenology (Xie et al. 2012). In a previous study we identified seven linkage groups in orchardgrass and showed the presence of QTLs and genes that affecting and controlling the variation for heading data. That map was intended as a test to determine if candidate genes for heading date coincided with QTL regions. Targeted enrichment reported herein produced orchardgrass sequence from three candidate genes for heading date: *Vrn1*, *Vrn3/Ft3*, and *Hd1*. Polymorphic primers were detected for all three genes, but due to a lack of mappable segregation ratios only the *Vrn3/Ft3* gene was mapped. Based on its position on LG3 of the *him271* parental map, the *Vrn3/Ft3* gene was not located within or near any QTL for late heading.

**Figure 1.** Linkage group 3 of the *D. glomerata himalayensis* parental map. The *Vrn3* gene was mapped in between the EST contig10375 and the AFLP markers.

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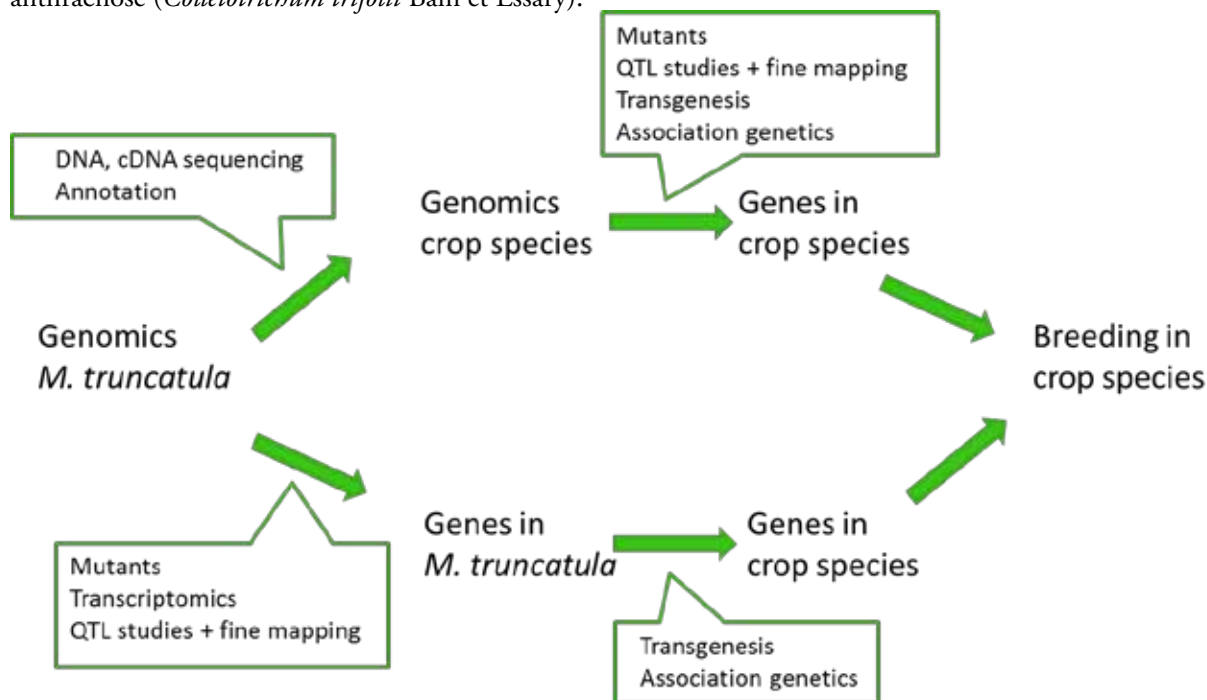
# Alfalfa breeding benefits from genetic analyses on *Medicago truncatula*

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Efforts in developing *M. truncatula* genetics and genomics resources were devoted while keeping in perspective the genetic improvement of related crop species such as alfalfa and clovers. The transfer of knowledge from *M. truncatula* to crop species is not simplistic. In order to improve the breeding programs, the detection of genes involved in variation for breeding traits is needed. Two non-exclusive strategies can be proposed. In the first one, the knowledge from *M. truncatula* genomics serves as a reference to develop the genomics of the crop species that is used to detect genes involved in traits, and these genes are becoming available to be incorporated into breeding programs (Figure 1). In the second one, *M. truncatula* genomics is valued by the detection, in the model species, of candidate genes that are tested for their effect in crop species (Figure 1). This second strategy was further explored considering two traits of agronomic interest in alfalfa: flowering date and stem length which is correlated to forage yield and resistance to anthracnose (*Colletotrichum trifolii* Bain et Essary).

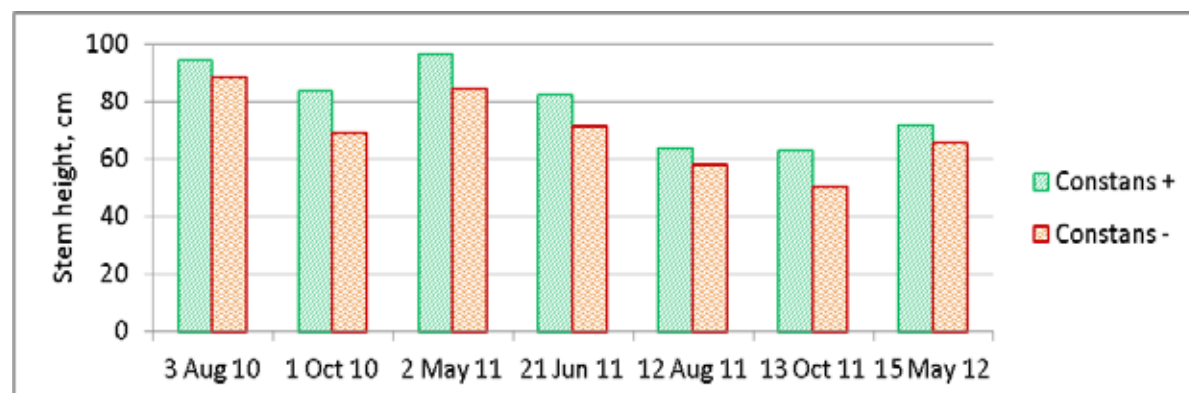


**Figure 1.** Two strategies to transfer genomics information from *M. truncatula* to the breeding program of a legume species. Several methodologies (boxes) are proposed to achieve each step.

In these two cases, the identification of genes in *M. truncatula* was based on QTL detection in mapping populations, followed by a fine mapping step. For flowering date and stem length, a major QTL was identified on the chromosome 7 and the confidence interval of the QTL was narrowed down using a fine mapping study (Julier et al. 2007; Pierre et al. 2008). Using an *in silico* analysis of the genes annotated in the QTL region and a literature review of the genes known to be involved in flowering date, six candidate genes were found. Among them, Constans-like gene contained sequence polymorphism and differential expression between the two parents was identified (Pierre et al. 2011). Similarly for anthracnose, a major QTL was found on chromosome 4 in a region that contained several resistance-like genes. Among them, the RCT1 gene was isolated and proved to induce resistance in RCT1-transformed alfalfa plants. The resistant *M. truncatula* genotypes showed an alternative splicing of intron 4 albeit the intron was correctly spliced out in susceptible genotypes (Yang et al. 2007; Yang et al. 2008).

The next step was to determine if these genes were involved in trait variation in alfalfa and evaluate their value to increase genetic progress in breeding programs. For the Constans-like gene, a population of 400 individuals (40 individuals from 10 varieties) was used for an association study to correlate sequence polymorphism with phenotypic

variation. By using SSR markers, this population was proved to have no genetic structure (Herrmann et al. 2008). All the plants were phenotyped for flowering date and stem length in two locations for four years, in isolated plant nurseries with three replications (clones). Two portions of the gene were sequenced, one covered most of the first intron and the beginning of the first intron and the other one included most of the second intron and a part of the second intron. Each portion was about 500 bp-long. Eight SNP having a frequency above 10% were identified, resulting in one SNP every 125 bp. In the same population, the sequencing of a neutral gene (glutamate synthase) showed 1 SNP every 30 bp. This difference in SNP frequency indicated that Constans-like gene is not neutral in alfalfa. From the 8 SNP, two of them explained up to 4% of the phenotypic variation, depending on the harvest and location. Two divergent populations were generated by polycrossing seven individuals with or without the positive alleles, respectively. Their evaluation in a spaced plant design over two years showed that the two populations differed for stem length by 7 to 15 cm in each harvest (Figure 2).



**Figure 2.** Stem length of the two polycrosses with (Constans +) and without (Constans -) the positive alleles of Constans-like gene, evaluated in a spaced plant nursery over 7 harvests.

For the RCT1 gene, a bulk segregant analysis was performed with the objective to compare the allele frequency between the bulks that included susceptible vs. resistance plants. Eight varieties were chosen, 100 plants were phenotyped in each variety and, among them, 15 susceptible and 15 resistant plants were identified. Eight pairs of bulks were thus generated. The whole gene (14 kb) was amplified for each genotype and the PCR products were bulked before sequencing on a next-generation sequencer (Roche 454). A gene assembly was performed with CAP2 on Unix for each bulk. Polymorphisms were too frequent in intron regions to align the sequences. Focusing on exons, five polymorphic regions were identified. The comparison of allele frequencies between bulks was unsuccessful: no polymorphic region accounted for the difference in resistance among the varieties. Possible scenarios are either the polymorphism related to resistance is located in non-coding regions, or another gene is involved in the resistance in alfalfa. We identified one infrequent polymorphism consisting of a deletion in the first exon around the ATG that was more frequent in the resistant plants compared to the susceptible plants. This deletion should induce a lack of function. Similarly in *M. truncatula*, the alternative splicing of intron 4 may limit the functioning of the protein. This deletion in ATG region could be tested for potential value to alfalfa breeding programs (Julier and Meusnier 2010; Julier et al. 2012).

These two examples show that genetic analyses in *M. truncatula* can be used to find genes involved in agronomic traits in crop species. These genes may explain phenotypic variation in alfalfa, as in the case of Constans-like gene, and may be used in breeding programs. The result is less clear for anthracnose resistance but further tests are needed to assess the role of RCT1 in anthracnose resistance in alfalfa.

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## The Lolium GenomeZipper – targeted use of grass genome resources for ryegrass genomics

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### Background

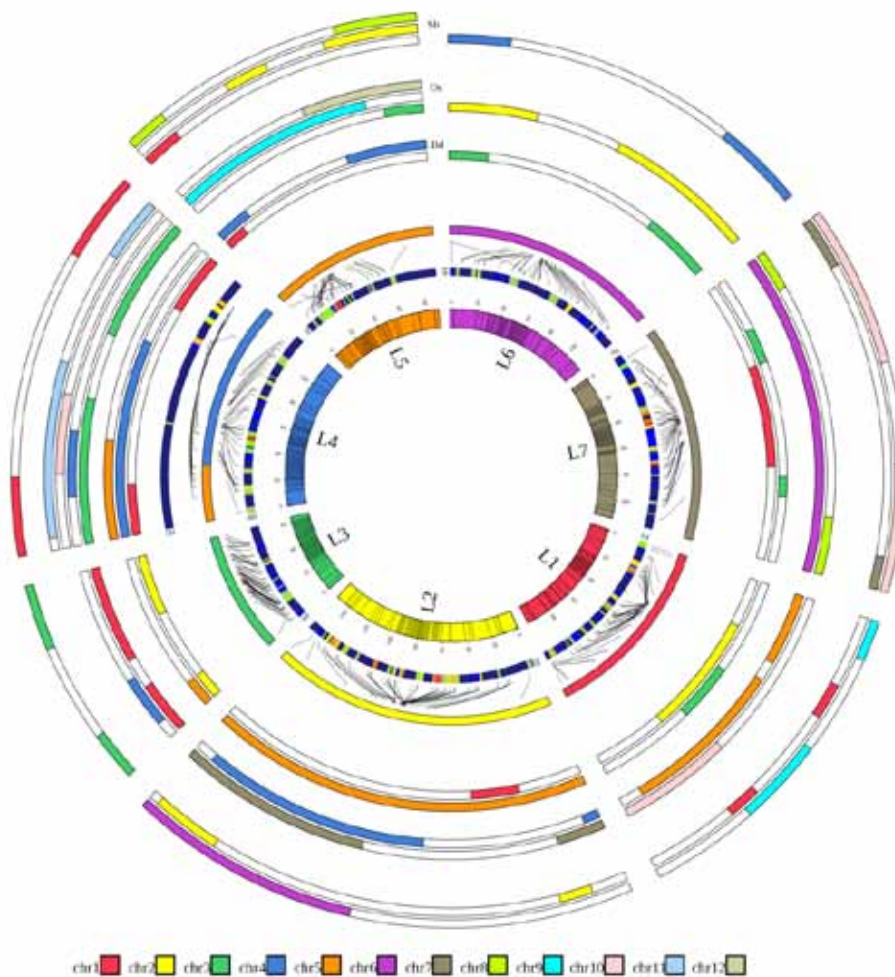
In the past decades, intense research efforts in model and major crop species has led to the establishment of whole genome sequences which constitute an important resource for genetic and genomic applications [1-4]. For forage and turf grass species, however, the development of whole genome sequences is hampered by the size and the complexity of their genomes. Thus, targeted use of grass genome sequence resources by comparative genomics provides a major opportunity for non-model species to efficiently explore genomic information for genetic and breeding applications. A novel approach incorporating cytogenetics, next generation sequencing and bioinformatics to systematically exploit synteny with model grasses was recently used in barley (*Hordeum vulgare* L.) to establish a genome-wide putative linear gene index of the barley genome. In this "artificial" barley genome, 21,766 barley genes were assigned to individual chromosome arms and assembled in a linear order [5]. Here, we have used a similar approach for perennial ryegrass (*Lolium perenne* L.) and report on a linear gene order model of the *Lolium* genome (the *Lolium* GenomeZipper) on the basis of synteny to barley, Brachypodium, rice and sorghum. In addition, we have characterised chromosomal rearrangements of syntenic genes between *Lolium*, barley and sequenced model grass species and applied the *Lolium* GenomeZipper for *in silico* prediction of the genomic location of previously unmapped *Lolium* genes.

### Results

A *Lolium* transcriptome map [6] served as scaffold to identify chromosomal arrangements of syntenic genes in sequenced grass species. A total of 762 EST-derived markers (between 79 and 154 on each linkage group, 109 on average) have been used to locate *Lolium* EST sequences on the linkage map. The total map length was 750 centiMorgan (cM), ranging from 63 cM on linkage group (LG) 3 to 151 cM on LG 2 (mean LG length of 107 cM) with an average marker distance less than 0.9 cM. Another 8,876 expressed *Lolium* genes of unknown chromosomal

origin were used for *in silico* mapping. For each of the seven *Lolium* LGs, the EST sequences of mapped DNA markers were compared against the barley artificial genome containing 21,766 ordered barley genes [5]. In total, 301 of 762 (40%) *Lolium* markers matched a barley full length (FL)-cDNA. The *Lolium* LGs and the corresponding barley chromosomes were mostly collinear, indicating a highly conserved gene order between these two species. A large-scale chromosomal translocation on *Lolium* LG 4 to Triticeae chromosomes 4 and 5 was found and its chromosome breaking point was further resolved.

Compared to *Brachypodium*, rice, and sorghum, a high degree of synteny and macro-collinearity between the genome of *Lolium* and sequenced grass species was found (Figure 1). This scaffold was then used to anchor a collection of expressed *Lolium* genes *in silico* to their predicted position in the *Lolium* genome. This resulted in the unambiguous assignment of 3,315 out of 8,876 previously unmapped *Lolium* genes to the respective chromosomes.



**Figure 1.** Syntenic relationship between *Lolium* and barley as well as deduced syntenic segments in *Brachypodium*, rice, and sorghum

Figure 1 includes five sets of circles: The inner circle represents the seven *Lolium* chromosomes (L1 to L7) scaled according to the size of the genetic map. Marked positions within the bars indicate the location of *Lolium* markers. In the next set including three circles, the schematic structure of the syntenic barley bridgehead is illustrated. For each *Lolium* chromosome, the barley artificial chromosome that was matched significantly by *Lolium* markers



is visualized as heatmaps highlighting the density of hits. Each match between a *Lolium* marker and a barley full length-cDNA sequence (as arranged in the artificial chromosomes) is indicated by black lines. The bars between the heatmap layers illustrate the syntenic barley blocks based on the sequence of the colour key, starting with chromosome 1 to chromosome 7. Moving outwards, the next three sets show the syntenic segments defined for the genomes of *Brachypodium* (Bd), rice (Os) and sorghum (Sb). Each bar represents an entire syntenic chromosome of one of the genomes. Coloured segments visualize that part of the chromosome which was defined as syntenic to *Lolium* via the barley bridge. Chromosomes are assigned according to the colour key.

Based on conserved regions between grass genomes, 2,438 barley full length-cDNAs and 2,758 syntenic genes of *Brachypodium*, 2,270 of rice, and 2,351 of sorghum were matched by 2,926 *Lolium* ESTs as evaluated by stringent best bidirectional hit sequence comparisons. In total, the *Lolium* GenomeZipper incorporates 4,035 gene loci (Table 1).

**Table 1.** General overview of the *Lolium* GenomeZipper

Number of	L1	L2	L3	L4	L5	L6	L7	$\Sigma$
Loci with <i>Lolium</i> EST evidence	565	690	636	586	500	487	571	4,035
<i>Lolium</i> markers matching Bd, Os or Sb genes	19	13	28	26	15	18	13	132
Anchored barley full length-cDNAs	395	408	400	341	277	276	341	2,438
Anchored <i>Lolium</i> ESTs via bi-directional hit	408	501	461	445	344	372	395	2,926
Anchored <i>Lolium</i> ESTs via first-best hit	476	558	538	384	408	425	463	3,252
Anchored <i>Brachypodium</i> genes	397	451	450	375	365	350	370	2,758
Anchored rice genes	322	408	403	313	205	311	308	2,270
Anchored sorghum genes	334	390	409	320	257	297	344	2,351

## Discussion

The *Lolium* GenomeZipper provides a high-resolution scaffold of the *Lolium* genome and offers the opportunity for a more detailed analysis of the organization and evolution of the *Lolium* genome. The integrative Zipper approach has emerged as a milestone for genome analysis that allows researchers to rapidly develop draft gene-decorated chromosomal templates even for large and complex grass genomes [5]. This is an important development since the size and complexity of the *Lolium* genome are major barriers towards developing a reference genome sequence in *Lolium*. Moreover, such chromosomal templates are instrumental for genome resequencing, genotyping by sequencing and large-scale marker development strategies in genome-wide association studies and genomics-based breeding concepts.

Apart from revising our understanding of the genomic relationship of *Lolium* to well described grass species, the *Lolium* GenomeZipper will be useful for a broad range of forage and turf grass species that are - so far - not well characterized. For *Poa*, *Dactylis* and *Phleum* species, for example, the *Lolium* GenomeZipper constitutes a unique tool for efficient development of markers at any genome position that underlie trait variation in *Lolium* and/or other major grass species such as barley, *Brachypodium*, rice and sorghum. As an example, multiple sequence alignments of genes conserved within Poaceae that have a well defined biological function can easily be generated by means of the *Lolium* GenomeZipper. Conserved regions within these sequence alignments can be identified and then used for primer design in order to isolate orthologs in the species of interest. This will greatly benefit linkage mapping-based QTL analysis or candidate gene-based association mapping in genetically more complex *Poa* and *Phleum* species where linkage mapping is generally difficult [7]. For other species such as *Festuca* spp. with considerably more EST or genomic sequence resources available, the present study provides the technological tools for the development of GenomeZippers in other forage and turf grass species, a straightforward approach to establish powerful tools for genome analysis. In the future, we envision using next generation transcriptome sequencing in uncharacterized forage and turf grass species and aligning *in silico* the assembled genes to the *Lolium* GenomeZipper, thereby very quickly obtaining high resolution maps.

## Conclusions

The Lolium GenomeZipper presented here is an ordered, information-rich scaffold of the Lolium genome and constitutes an important tool for the assignment of candidate genes to QTL, for map-based cloning, functional genomics and the Lolium genome assembly. Moreover, GenomeZipper-based comparative genomics holds the key to unlock the genomes of the most important forage and turf grass species.

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## Molecular breeding for the improvement of winter hardiness in perennial ryegrass (*Lolium perenne* L.) by introgression of genes from meadow fescue (*Festuca pratensis* Huds.)

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## Introduction

Perennial ryegrass (*Lolium perenne* L.) is often used for grazing in temperate grasslands with its good regrowth and high nutritive value, whereas meadow fescue (*Festuca pratensis* Huds.) has superior tolerance to abiotic stresses such as freezing, and thus has good overwinter survival in grasslands. To combine the attractive properties of both species in one genotype, their inter-specific hybrids, festulolium has been bred since the 1970s. In this decade, quantitative trait loci (QTL) for winter hardiness-related traits have been identified in perennial ryegrass and meadow fescue (Yamada et al. 2004; Alm et al. 2011; Bartos et al. 2011). A few *Festuca* chromosome segments able to increase tolerance to freezing were identified in a *L. multiflorum* background by genomic *in situ* hybridization (GISH) (Kosmala et al. 2006; 2007). However, the genetic factors involved in differential winter hardiness-related traits in perennial ryegrass and meadow fescue are poorly understood. In this study, to improve the winter hardiness of perennial ryegrass using genes from meadow fescue by molecular breeding, introgression mapping of winter hardiness-related traits were performed using DNA markers. As another approach to identify candidate genes for winter hardiness-related QTLs between the two species, a comparative transcriptome study was conducted during cold acclimation.

## Introgression mapping of winter hardiness

Three triploid hybrids (one F1 plant between a diploid meadow fescue plant from “Makibasake” and a tetraploid perennial ryegrass plant from “Pokoro” and two of its progenies after backcrossing with diploid perennial ryegrass) were backcrossed with diploid perennial ryegrass plants from the strain “Yatsugatake D-12.” Progenies generated from the three populations estimated as being diploid plants (n = 203) by ploidy analyzer were used for further analysis. Intron-flanking EST markers with locating chromosome information in *Lolium/Festuca* were used

to identify introduced alleles from meadow fescue (Tamura et al. 2009; 2012). Genotyping of 91 markers selected equivalently from all chromosomes indicated that 63% of plants in the three populations had (partial) chromosome fragments derived from meadow fescue. In some genotypes, introduction of meadow fescue-derived chromosomal fragments into perennial ryegrass was also confirmed by GISH analysis.

The freezing tolerance of cold-acclimated crown tissues was evaluated by scoring the degree of regrowth after freezing (0: dead to 9: completely alive). Mean freezing tolerance scores at  $-12.5^{\circ}\text{C}$  were 3.1 for “Yatsugatake D-12” and 6.1 for “Makibasakae.” Backcrossed populations showed wide variations in freezing tolerance, and the mean scores in the three populations (2.4) were lower than those in “Yatsugatake D-12.” Of the 12 selected genotypes that showed high freezing tolerance, lethal freezing temperature (LT50) in crown tissues was evaluated. The lowest LT50 was  $-17.0^{\circ}\text{C}$  in genotype A12-24-91. This was significantly lower than the  $-12.9^{\circ}\text{C}$  LT50 in D12F18, a diploid perennial ryegrass genotype used for backcrossing, and much higher than the  $-26.0^{\circ}\text{C}$  LT50 in Makibasakae-K1, a meadow fescue genotype used for triploid hybrids (Fig. 1). The similarly low LT50 of high freezing tolerant introgression genotypes and a triploid hybrid ( $-15.2^{\circ}\text{C}$ ) suggests that introgression of single or a few chromosomal fragment(s) from meadow fescue could increase freezing tolerance in perennial ryegrass to at least the hybrid level.

Winter survival was evaluated from 2009 to 2011 in a field in Sapporo, Japan, where winter soils are covered by snow and not frozen. After two years of wintering, almost all genotypes of “Yatsugatake D-12” evaluated were completely dead, whereas some backcrossed genotypes survived. However, no genotype showed scores for winter survival and early spring yields higher than “Makibasakae” plants. On the marker loci with a meadow fescue allele frequency higher than 5% in each population (a mean of 44 % of all markers), the association between the allele type (presence or absence of a meadow fescue allele) and winter hardiness-related traits (freezing tolerance, winter survival, and early spring yields) were statistically analyzed. We found some marker loci showing significant positive effects from meadow fescue alleles for winter survival and early spring yields on chromosome 7. In particular, introducing a meadow fescue allele of the rice Os06g13810 homoeologue showed significant positive effects on winter survival scores or early spring yields in all three populations. On the contrary, negative effects of meadow fescue alleles were found at several marker loci on chromosome 4, including for winter survival and early spring yields after the first winter. A significant positive association between a meadow fescue allele and freezing tolerance was not found for any marker loci. Marker loci showing positive and negative effects of the meadow fescue allele for winter hardiness in this study could be more precisely investigated by backcrossing of the introgression genotypes, which would be useful information for the selection of superior genotypes. To scan for useful introgression across the whole genome, genetic analysis using populations with higher frequency of meadow fescue alleles through the whole genome is required, and this is the next challenge.



**Figure 1.** Regrowth after freezing ( $-15^{\circ}\text{C}$ ) of a meadow fescue genotype, Makibasakae-K1 (a), an introgression genotype with the highest freezing tolerance examined in this study, A12-24-91 (b), and D12F15, a perennial ryegrass (c).

### Comparative transcriptome analysis during cold acclimation in perennial ryegrass and meadow fescue

To identify differentially expressed genes in perennial ryegrass and meadow fescue during cold acclimation, an inter-specific comparative transcriptome study was performed by the mRNA-seq approach (Tamura and Yonemaru 2010). cDNA or inter-specific subtractive cDNA between perennial ryegrass and meadow fescue were constructed from cold acclimated crowns, which were sequenced using GS FLX at a small scale (5,000–10,000 reads). Generated reads were clustered based on sequence homology among and within species. We compared the read number in each cluster and validated the expression level in diverse genotypes of both species by quantitative RT-PCR using primer sets designed to amplify the conserved sequences. In perennial ryegrass, jasmonate-

induced protein and germin-like protein genes showed significantly higher expression compared with meadow fescue. Alternatively in meadow fescue, several stress tolerance-related gene expression were significantly higher than those in perennial ryegrass; particularly, the expression of ice recrystallization inhibition protein and metallothionein type-2 whose involvement in freezing tolerance has been reported previously. These highly expressed genes in meadow fescue



were transcriptionally upregulated by a low temperature (4°C). Increased transcript levels during cold acclimation were also confirmed in the field in Sapporo, Japan, from October to mid December in 2011. To clarify the relationship between gene expression levels during cold acclimation and winter hardiness including freezing tolerance, evaluation of introgression genotypes having meadow fescue alleles of candidate genes in a background genome from perennial ryegrass is ongoing.

## Conclusion

Diploid hybrids between perennial ryegrass and meadow fescue are sterile, and thus, application of linkage genetic analysis is quite limited in *festulolium*. Therefore, in addition to genetic mapping using introgression populations, a candidate gene approach is an important alternate approach. This could be accomplished by application of QTL information obtained by linkage genetic analysis of each species and information from functional genomics and -omics studies, such as the inter-species comparative transcriptome analysis performed in this study. A study using a combination of introgression mapping and a candidate gene approach would provide useful selection markers for *Lolium/Festuca* introgression breeding.

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## SNP discovery and candidate gene-based association mapping of forage quality traits in perennial ryegrass

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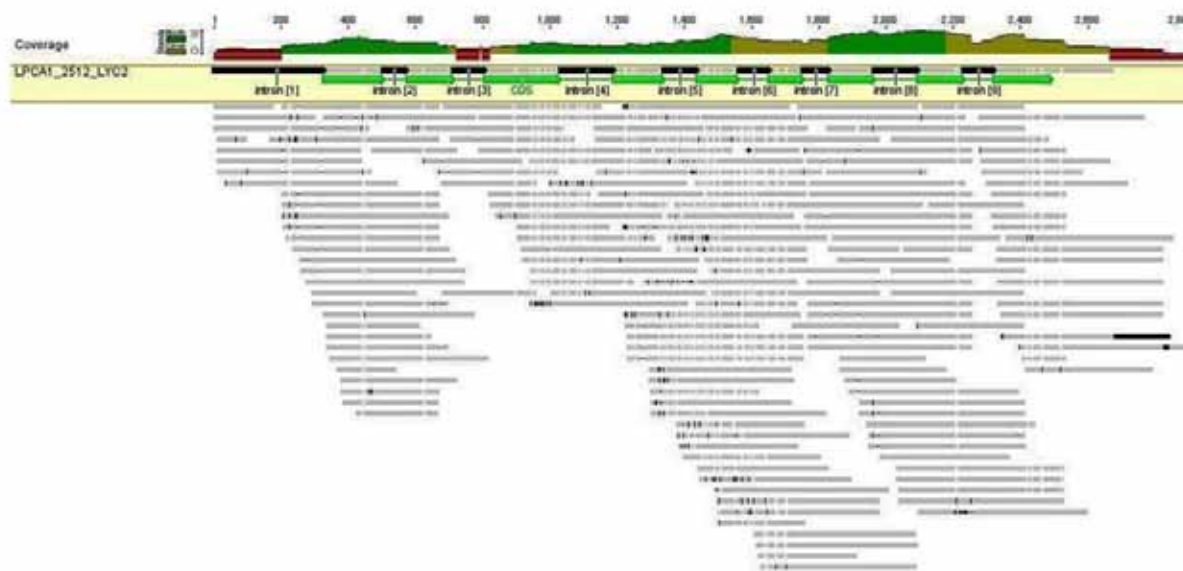
## Abstract

Repetitive DNA provides a challenge for performing targeted DNA capture in plants. An array based method and an in-solution procedure (MYselect) were compared for optimal targeted DNA capture of 1300 genes in perennial ryegrass. The MYselect procedure gave superior results with >50% of reads on target. This protocol together with 454 sequencing is being used to identify single nucleotide polymorphisms (SNPs) and determine gene haplotypes from plants in elite breeding lines and diverse wild collections. Targeted DNA capture, in combination with barcoding of samples, will allow for a genotype-by-sequencing (GBS) approach. Several genes have been identified where loss-of-function gives an improved trait phenotype. Mutations potentially causing loss-of-function were identified by sequencing amplicons derived from DNA pools. In an alternate approach, rapid decay of linkage disequilibrium in

perennial ryegrass is ideal for candidate gene-based association studies to identify functional nucleotide polymorphisms (FNPs) responsible for trait variation. Nineteen candidate genes in the lignin and fructan metabolic pathways generally displayed rapid decay of linkage disequilibrium characterised by  $r^2$  values below 0.2 within distances of 1kb. A candidate gene in the fructan pathway displayed copy number variation. Subsequent association tests performed in a diverse association mapping population provided some evidence of correlation with fructan content but are inconclusive because population structure did not allow for a definitive test.

### Targeted genomic DNA capture

Plants with larger genomes, such as perennial ryegrass (*Lolium perenne*), possess abundant repetitive DNA which interferes with targeted DNA capture techniques. Therefore two different methods were compared for optimal genomic DNA capture of 1300 genes. The array-based method used gave only 0.8% of reads on target with non-target sequences predominantly belonging to repetitive DNA such as retrotransposons and simple sequence repeats (SSRs). A second approach used the MYselect method (renamed MYbaits from MYcroarray; <http://www.mycroarray.com/mybaits>) which is a solution-based enrichment technique using biotinylated RNA baits. Target sequences were repeat-masked with repetitive sequences from the array experiment and the Poaceae grass family repeat database. After capture, sequencing was performed on a Roche 454 FLX or GS junior sequencer. Optimal results were obtained when a second capture was performed on the primary capture. Using this method 50-75% of reads were routinely on target. Target bias was observed and attributed to either PCR bias of some target sequences during library amplification steps or failure of the probe to bind on intron-exon boundaries, since approximately 40% of the sequences were based on ESTs. Novel DNA sequence was obtained from introns (Figure 1) or the 5' or 3' end of the genes targeted. Probes were also able to capture paralogous genes for many of the sequences targeted. This targeted DNA capture method, combined with custom barcoding of samples and the use of NextGen sequencing platforms, increases the feasibility of a genotype-by-sequencing approach.



**Figure 1.** Capture of novel sequence from 9 introns using an EST sequence for probe design.

### Identification of naturally occurring gene mutations

Loss-of-function is desirable for some genes to give an enhanced phenotype. For example, several lignin biosynthetic genes (CAD, COMT, CCoAOMT) increase feed conversion efficiency by altering digestibility when down-regulated in forages (Chen et al. 2003 & 2004; Guo et al. 2001). Using a combination of DNA pooling and 454 amplicon sequencing, 18 genes were screened for mutations in 500 plants. Replicate PCR analyses were included to enable the identification of DNA polymerase errors. Uniform coverage of amplicons was achieved and each plant within a pool was represented approximately five-fold per amplicon. Seven putative loss-of-function mutations were identified in

four target genes. Five of the mutations were predicted to cause premature termination of the protein and two were deletions in conserved protein motifs. A previously known mutation was detected at the expected frequency level proving the reliability of the method and the representativeness of the germplasm pool. The mutations identified were all at very low frequency ranging from 0.5–4%. Plant breeding crosses will be required in most cases to create and evaluate homozygous lines, given the rarity of the mutations.

### Candidate gene-based association mapping

Linkage disequilibrium (LD) decays rapidly in most perennial ryegrass genes if the population evaluated is a diverse representation of the species. For example genes from the lignin and fructan biosynthetic pathways are typical with LD characterised by  $r^2$  values below 0.2 within 1kb. This characteristic allows for the very fine mapping, and possible identification, of the functional nucleotide polymorphisms (FNPs) responsible for trait variation. High expression levels of the candidate gene Fr1 are associated with higher fructan content. SNP discovery was performed on this gene using a panel of 16 plants. Two distinct isoforms (Fr1a & Fr1b) were identified based on DNA sequence identity. Through a combination of evidence from genetic mapping and haplotyping of additional plants, it was hypothesized that Fr1b is a tandem duplication of Fr1a. As a result, Fr1b may be either hemizygous or homozygous. A genotyping assay was designed and 326 plants from an association mapping population evaluated. Most plants in the population only had the Fr1a locus and were missing Fr1b. The association mapping population was measured for fructan content over several seasons. An association was detected for higher fructan content in one season by ANOVA analysis but when population structure was accounted for the association proved spurious. Breeding pools that are being evaluated for fructan content will be monitored for any frequency shifts of the 3 genotype classes. This candidate gene-based association mapping approach will also be used for additional genes in the fructan and lignin biosynthetic pathways.

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## Condensed Tannin Expression in Legumes

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Legumes are a large and extremely diverse family, containing over 700 legume genera and 20,000 species and are second only to grasses in importance to human and livestock. The two larger clades, Hologalegina and the phaseoloid/milletooid group include some of the most important forage legumes, including *Medicago*, *Trifolium*, *Lotus*, *Hedysarum coronarium* (sulla), and *Onobrychis* (sainfoin) species. Many of these species play an important role in agricultural forage, by providing a valuable high nutritive feed source. The presence or absence of Condensed Tannins (CT) varies greatly within such forage legume species. Foliar CT are present in forage plants such as *Lotus corniculatus* (Birdsfoot trefoil), and *Onobrychis viciifolia* (Sainfoin) but such species often show poor persistence under grazing. Foliar CT bind reversibly with forage proteins within the rumen and thus reduce protein degradation. This results in an increase in protein outflow from the rumen, leading to improved protein utilization by ruminants (McNabb et al. 1996). Forages containing moderate amounts of CT therefore improve the nutrition and production qualities for grazing livestock, including animal productivity, animal health and environmental aspects, while higher levels of CT in the diet of ruminants decrease palatability and feed intake (Aerts et al. 1999).

In contrast, forage legumes that can persist in temperate grazing systems, such as *Trifolium repens* (white clover), and *Medicago sativa* (alfalfa), have only negligible amounts of CT in leaves. Alfalfa is the most important and widely grown legume crop in pastoral agriculture, especially in Australia and USA. In New Zealand where the grazing system is based upon mixed pastures, the predominant legume is white clover (*Trifolium repens*), which represents roughly

20%, while the major portion consists of grasses such as perennial ryegrass (*Lolium perenne*). This lack of CT causes unique problems including bloat, inefficient protein utilisation and adverse loss of nitrogen (N) into the environment. Inefficient nitrogen utilisation by the rumen, especially of the high-protein forages, compromises animal production and health. Additionally other significant environmental issues pertaining to greenhouse gas (GHG) emissions need to be addressed. Urinary nitrogen lost to the environment is rapidly converted to N<sub>2</sub>O, a potent GHG. Grazing ruminants are also responsible for the production of GHG emissions in the form of methane and nitrous oxide. All ruminants produce methane gas from rumen fermentation and this represents an additional loss of feed energy (2–12%) from the diet (Woodward et al. 2001).

## Biochemistry of Condensed Tannins

Foliar CT, also known as proanthocyanidins, are polymeric flavonoids, derived from the phenylpropanoid pathway and share a large portion of the anthocyanin pathway enzyme steps. Biosynthesis and accumulation of CT is still not fully characterized in legumes, but major advances have elucidated several steps in this complex pathway. One of the most desired characteristics for white clover and alfalfa cultivars is the ability to accumulate CT in leaves. Increased CT levels in clover would significantly enhance animal health and performance as well as reduce greenhouse gas emissions from pasture animals. Several approaches, including screening gene pools and mutagenesis, have failed to provide a white clover, lucerne or red clover containing foliar CT (Woodfield et al. 1998). Moreover, attempts, using traditional breeding techniques, have allowed development of high floral tannin cultivars. However, since an apical bud can initiate either a flower or a stolon but not both, increased flower number and CT production concomitantly reduced stolon numbers, resulting in inadequate agronomic performance and low persistence, when compared to elite cultivars. (Burggraaf et al. 2006).

One of our main research interests in white clover focuses on understanding and manipulating CT biosynthesis. We directed efforts towards attempting to alter the spatial and temporal accumulation patterns present in white clover and alfalfa in a way that would allow the production of CT in the leaves (Hancock et al 2012). Although white clover (*Trifolium repens*) is a high-quality pasture legume, it produces negligible levels of CT, located only in trichomes on the abaxial epidermal layers of *T. repens* leaves.

The clover genus *Trifolium* L. is one of the largest genera in the family Leguminosae with ca. 255 species (Ellison et al. 2006). Only two species, namely *T. arvense* (rabbits foot clover) and *T. affine* (*T. preslii* Boiss. Is), are known to accumulate significant levels of CT in leaves. One of these, *T. arvense*, which is a frequent and important component in low-altitude, semi-arid tussock grasslands in New Zealand, was investigated further. Staining of *T. arvense* tissues from wild-type plants with DMACA (p-dimethylaminocinnamaldehyde; Li et al. 1996) showed that CT accumulates in the epidermal layers of the entire leaf lamina.

## Genetic Modification of Condensed Tannins

To isolate R2R3-MYB transcription factors potentially involved in CT biosynthesis in *T. arvense*, we constructed a cDNA library from leaves of *T. arvense* and identified a number of MYB factors. Two *T. arvense* cDNAs coded for protein sequences fell within the MYB clades NO8 and NO9 (Stracke et al. 2001), whose members include those known to activate CT or anthocyanin biosynthesis. Full-length putative orthologues from genomic DNA were also isolated from the *Trifolium* species *T. arvense*, *T. affine*, *T. repens*, *T. occidentale*, and the *Medicago* species *M. sativa*, and *M. truncatula*.

Expression analysis using cDNA libraries from callus, immature and mature leaf tissues revealed that TaMYB14 was expressed in immature and mature *T. arvense* leaves, but not in callus tissues which do not accumulate CT. Transcripts of MYB14 orthologues were also detected in cDNA libraries from meristematic leaf tissues, trichomes and floral tissues from *T. repens* and *T. occidentale*, but not in young and mature leaf tissues, stolons, internodes, roots, and petioles. No MYB14 transcripts were detected in a red-leaf *T. repens* line ('Isabelle') which accumulates high levels of anthocyanins in foliar tissues, but does not accumulate foliar CT except in trichomes and flowers. Expression of MYB14 was paralleled by the expression of the structural genes ANR and LAR which code for enzymes catalyzing the last steps of PA monomer synthesis. This indicates that expression of MYB14 coincides with CT biosynthesis, and that CT biosynthesis is activated in *T. arvense* in both immature and mature tissues, while it is only activated in meristematic leaf and floral tissues of *T. repens*.

A segment of the gene was used in a silencing vector and transformed into *T. arvense*. The plants expressing this construct had diminished or trace amounts of CT. Hence RNAi silencing of TaMYB14 resulted in almost complete

cessation of CT biosynthesis in *T. arvense*, Neither wild type nor silenced *T. arvense* callus tissues contained CT; leaves from regenerated wild type plants stained positive, while leaves from regenerated TaMYB14-silenced plants showed only a light blue staining in the leaves, indicating that CT biosynthesis was severely reduced in TaMYB14-silenced plants. Extracts of wild type *T. arvense* leaves contained high levels of CT monomers, mainly catechin and, to a lesser extent, gallicocatechin, as well as PC:PC (procyanidin based), PC:PD (prodelphinidin based) and PD:PD dimers and trimers of the various PC:PD combinations. No higher DP (degrees of polymerisation) polymers were seen in LC-MS/MS (Liquid chromatography–mass spectrometry) scans, but further work on concentrated acetone fractions revealed the presence of up to DP6 CTs in wild type *T. arvense* leaves. In contrast, only traces of CT monomers, but no dimers or trimers were detected in leaf extracts from silenced plants, indicating that CT biosynthesis had been severely reduced by TaMYB14 silencing, and that the expression of TaMYB14 is required for CT synthesis in *T. arvense*.

In addition, the gene was cloned into a binary vector (driven by 35S promoter) and transformed into tobacco, alfalfa and white clover lines. The resulting transgenic plants accumulated CT in foliar leaf tissue at high levels. Analysis of DMACA and LC-MS/MS in tobacco plants transformed with the CaMV35S::TaMYB14 construct stained positive for CT with DMACA, while untransformed tobacco plants did not, indicating CT biosynthesis occurred due to the expression of TaMYB14. Monomers of CT (mainly epicatechin with small amounts of epigallocatechin and gallicocatechin), PC:PC dimers and trace levels of trimers were detected by LC-MS/MS analysis of flavonoid extracts from TaMYB14 expressing tobacco plants, while CT were undetectable in control plants. These results provide strong evidence that TaMYB14 is able to up-regulate the biosynthesis of CT, even in plants phylogenetically distant from *T. arvense*, and indicates that up-regulation of CT biosynthesis competes with substrates for anthocyanin biosynthesis or accumulation as indicated in transformed high anthocyanin tobacco lines.

In alfalfa, plants were also transformed with TaMYB14 under the control of the CaMV35S promoter. Leaves from non-transformed wild type plants stained positive with DMACA in the trichomes on the abaxial leaf layers only, while plants transformed with TaMYB14 stained positive in epidermal leaf cells as well. The presence of CT monomers (epicatechin and catechin), PC:PC dimers, PC:PC:PC and PC:PC:PD trimers, and trace levels of tetramers in leaf extracts of *M. sativa* plants transformed with the TaMYB14 construct was confirmed by LC-MS/MS analysis, while CT were undetectable in control plants.

White clover plants were also transformed with TaMYB14 under the control of the CaMV35S promoter; and the presence and expression of the transgene was confirmed by (RT)-PCR. Leaves from regenerated plantlets were screened for CT accumulation using DMACA staining and a number of plants transformed with TaMYB14 tested positive. Leaves from non-transformed wild type plants showed, as expected, positive DMACA staining in the trichomes on the abaxial leaf layers only, while plants transformed with TaMYB14 accumulated CTs in epidermal leaf cells as well as in trichomes. Microscopic examination under higher magnification revealed that stomatal guard cells as well as mesophyll cells stained positive with DMACA. Staining in trichome tier and apical cells was much stronger compared to wild type trichomes, and staining of spongy mesophyll cells indicated CT accumulation in multiple vacuole-like organelles. In some transgenic plants CT were also detected in root and petiole cells. A number of plants appeared to accumulate very high levels of foliar CTs, indicated by almost black staining with DMACA; however, all of these plants died before sufficient material for LC-MS/MS analyses could be harvested. These results indicate that the constitutive expression of TaMYB14 results in the ectopic production and accumulation of CT in foliar (and other) tissues and cell layers of *T. repens* which normally do not accumulate CT (shown below; DMACA CT staining of leaves from wild type and 3 transgenic plants).



**Figure 1.** (A) DMACA stained white clover leaves, showing wild type (left) and leaves from three transgenic plants stained tannin positive; (B) magnified positive white clover leaf showing vacuoles containing tannin.

To confirm the presence of CT units in *T. repens* leaves expressing TaMYB14, leaf extracts were analysed by

LC-MS/MS. No CT units were detected in wild type *T. repens* leaves, while CT monomers, dimers, as well as trace levels of trimers were detected in *T. repens* plants transformed with the CaMV35S::TaMYB14 construct. The major monomer detected was epigallocatechin, with 10-fold lower levels of epicatechin and galocatechin, and only traces of catechin detected. The dimers and trimers detected were PC and PD or all PD based, consistent with the observation of both PC and PD monomers in transformed *T. repens* leaves. These results provide evidence for TaMYB14 being able to activate the biosynthesis and accumulation of CT in white clover foliar tissues.

To analyse the effects of TaMYB14 on the expression of genes coding for enzymes of the phenylpropanoid pathway and a putative MATE-like CT transporter, RNA was isolated from leaves of white clover plants constitutively expressing TaMYB14. No significant differences in the expression of TrF3H and TrFLS in transgenic TaMYB14 compared to wild type *T. repens* leaves were detected. Expression of TrCHS, TrF3'5', TrDFR, and TrANS were up-regulated in plants transformed with the CaMV35S::TaMYB14 construct compared to wild type plants with a very strong induction of TrF3'5' (>600-fold). Expression of the putative CT-specific genes TrANR, TrLAR, and a transporter involved in the transport of CT units, TrMATE, could only be detected in leaves from plants constitutively expressing TaMYB14. Therefore, TaMYB14 expression is necessary and sufficient to up-regulate both early and late steps of the phenylpropanoid pathway and to induce CT biosynthesis.

## Conclusions

Our results demonstrate that expression of the R2R3-MYB transcription factor TaMYB14 is necessary and sufficient to up-regulate genes of the CT pathway and to activate the synthesis and accumulation of CTs in leaves of *Nicotiana tabacum*, *M. sativa*, and *T. repens*. *T. repens* plants constitutively expressing TaMYB14 synthesized and accumulated CTs in leaves up to 1.8 % dry matter in surviving white clover. Targeted LC-MS/MS analysis identified foliar CTs up to six degrees of polymerization in leaf extracts. Hence, genetically modified *M. sativa* and *T. repens* plants expressing TaMYB14 provide a viable option for mitigating certain animal health and environmental issues present in pastoral agriculture based farming systems.

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# High-energy perennial ryegrasses could provide economic value to dairy farmers in temperate Australia

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## Introduction

Animal production is the ultimate measure of feeding value (Paterson et al. 1994). Maintaining or improving feeding value to support animal growth or milk production is an important goal for pasture plant breeders and livestock producers. Despite this, pasture plant breeders have traditionally focussed most efforts on improving plant yield traits (Bathgate et al. 2005). Increasing the water soluble carbohydrate (WSC) concentration of pasture could be one way to improve feeding values. Genetic progress has been made increasing WSC concentrations using traditional selective breeding in perennial ryegrass (*Lolium perenne*) (Humphreys 1989). These genetic improvements have shown potential to increase milk production in cows (Miller et al. 2001) and growth of lambs (Lee et al. 2001). However, the expression of the trait has been inconsistent, particularly in the Australian environment (Francis et al. 2004).

Transgenic perennial ryegrass plants with enhanced fructan yields have been generated through the expression of chimeric fructosyltransferases under control of light-regulated promoters (Spangenberg et al. 2011; Spangenberg et al. 2012) with an aim to increase energy concentrations by over one megajoule of metabolisable energy per kilogram of dry matter, MJME/kg DM (Dairy Futures CRC 2011). This paper assesses the potential economic value for a range of changes in perennial ryegrass energy concentration on two contrasting pasture based dairy farm trials in temperate Australia.

## Methods

Data from two contrasting environments were used to estimate the economic value of changing energy concentration of perennial ryegrass consumed by dairy cows. This included the low summer-rainfall region of Terang, in south-west Victoria (36 dairy cows on 14-16 ha of land, calving in May-June) and the high summer-rainfall region of Elliott, in northern Tasmania (70 dairy cows on 16 ha of land, calving in early July). Perennial ryegrass intake was limited to home-grown sources as either forage or silage. The replacement cost method (Sinden & Thampapillai 1995) was used to estimate the economic value of having more energy available on the farm through an increase in perennial ryegrass energy concentration. Mean monthly barley prices in AU\$ per MJME (utilised) were used as an estimate of the cost of replacing pasture energy.

Increasing perennial ryegrass energy concentration was assumed to have no adverse effects on dry matter yield, pasture survival, or rumen function of dairy cows. No additional costs were assumed to be incurred when the high-energy perennial ryegrasses were chosen as a full pasture seed mix instead of a 'standard' perennial ryegrass variety. The model was not designed to maximise farm profit or productivity when the additional energy from high-energy perennial ryegrass was included in the system.

Economic values for changes in energy concentration of perennial ryegrass were first calculated for the 2009-10 season. Then, a range of years was used to assess how inter-annual variability affected the results. Six milking seasons (2005 to 2011) were used from Terang and four milking seasons from Elliott (2003 to 2007). A different range of years were chosen from each trial in order to capture the maximum number of years' data without significant changes to management.

Six scenarios for each trial were simulated. For Terang (T) and Elliott (E), this included '0.5B', '0.5H', '1.0B', '1.0H', '1.5B' and '1.5H'. Where the number indicated the change in energy concentration (in MJME/kg DM) and the suffix letter indicated whether the 'base' ('B') or '20% higher' ('H') price of barley was used. This provided for a sensitivity evaluation of the replacement cost of energy. The economic value for high-energy perennial ryegrass was calculated as the summation of dry matter intake as pasture and silage per hectare multiplied by the assumed change in energy concentration and the replacement cost of energy.

Economic values were differentiated between summer, autumn, winter and spring for the 2009-10 season but not when several years were simulated using Monte Carlo simulation. Economic values were calculated with changes

to the main variables including pasture intake, changes in energy concentration and the cost of barley energy. Results were analysed from 10,000 iterations of Monte Carlo simulation using multiple years' data. The @RISK program (version 5.7 from Palisade Corporation) was used to estimate the distribution of potential economic values for the two trials from each simulation.

### Results and Discussion

Results from one year of data (Figure 1) showed significant economic value for a one unit change in energy concentration (1 MJME/kg DM). A 1 MJME/kg DM change in energy concentration was equivalent to \$AU 217/ha economic value for Terang and \$AU 206/ha for Elliott in the 2009-10 milking season. Spring made the greatest contribution to overall economic value in both trials as higher pasture availability facilitated higher pasture consumption per hectare.

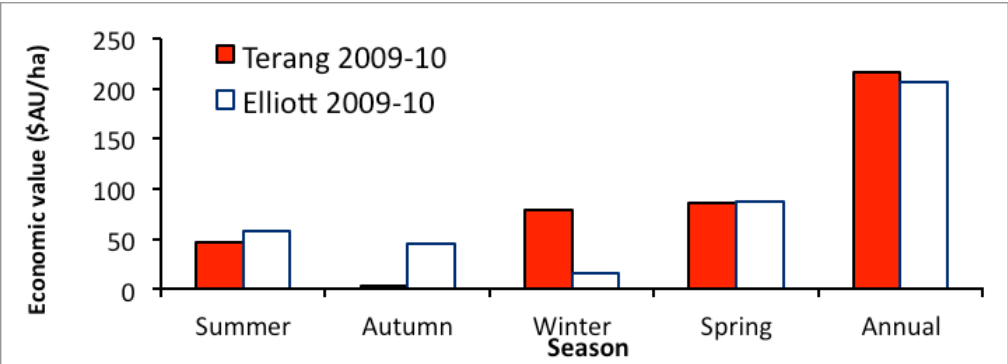


Figure 1: Potential economic value of high-energy perennial ryegrass for one milking season for a one unit (MJ metabolisable energy per kg dry matter) increase in energy concentration for the Terang and Elliott dairy trial data. Economic value is defined as the increase in energy concentration of high-energy perennial ryegrass over 'standard' perennial ryegrass.

Based on Monte Carlo simulation using several years' data (Figure 2), Elliott had higher economic values and greater absolute ranges in economic values compared to Terang (based on the difference between the 10<sup>th</sup> and 90<sup>th</sup> percentiles) over multiple years. For example, Elliott had \$AU281/ha higher mean annual economic value over the 2003-07 period compared to Terang when energy concentration changed by one unit. The mean economic value for a one unit change in energy concentration at Terang was \$AU204/ha per annum for 2005-2011.

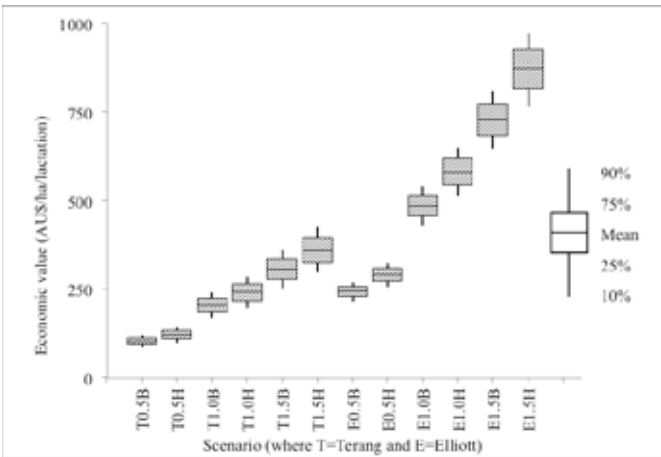


Figure 2: Terang (T) and Elliott (E) economic value means and ranges with 0.5, 1.0 and 1.5 MJME/kg DM changes in energy concentration of perennial ryegrass using base (B) and 20% higher (H) barley prices from 10,000 iterations of Monte Carlo simulation.



## Conclusions

Transgenic perennial ryegrass plants with higher water soluble carbohydrate concentrations could increase the mean energy concentrations of perennial ryegrass pastures. Estimates of the value of a one unit increase in energy concentration for perennial ryegrass were \$217 (Terang 'summer dry' in Victoria) and \$206/ha (Elliott 'high summer rainfall' in Tasmania) for the lactation period based on 2009-10 data. The scale of the potential benefits quantified in this study provides justification for more detailed modelling of management practices which capture greater advantage from the high-energy perennial ryegrasses.

## Acknowledgements

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## Gene Expression and Metabolite Analysis of Endophyte-infected and Endophyte-free Tall Fescue Clone Pairs Under Water Deficit Conditions

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## Introduction

The predominant forage of the eastern United States is tall fescue (*Lolium arundinaceum* = *Schedonorus arundinaceus* = *Festuca arundinacea*), which shows remarkable adaptability over the entire C<sub>3</sub>-C<sub>4</sub> transition zone. Tall fescue/endophyte symbiosis is important in U.S. agriculture because it represents a key component of forage systems in a large portion of the country (Zhuang et al. 2005). Tall fescue and related grasses are also highly important in soil conservation and strip-mine reclamation, and as a turf for yards and athletic fields. A significant factor in the exceptional fitness of tall fescue is its seed-transmissible symbiont, the fungal endophyte *Neotyphodium coenophialum*. The most common *N. coenophialum* strains produce ergot alkaloids and can cause episodes of "fescue toxicosis" to grazing livestock (Hoveland, 1993; Schardl et al. 2006; Thompson and Stuedemann, 1993). Removal of the endophyte, though feasible, is not a preferred solution because of its importance in host fitness characteristics.

*N. coenophialum* is disseminated only by seed, so the life cycle is relatively simple (Bacon and Siegel, 1988; Wely et al. 1986). Hyphae are present within the stem apex region of plants from the time that the embryo has matured (Hinton and Bacon, 1985). The endophyte colonizes developing seeds after anthesis, where it remains until the seed are planted and germinate (though it can die in prolonged or improper storage). Upon seed germination the hyphae grow along with the cells of the stem apex region of the embryo and infect new leaves and axillary buds, from which new tillers develop. The hyphae also colonize the inflorescence when it begins development at the tip of the stem

apex. The fungus moves from the germinating seed into the seedling, and colonizes mainly leaf sheaths, meristems, and internodes of elongating stems, but not roots. From the host plant the endophyte receives nutrients, protection, and vehicles for dissemination (tillers and seeds), whereas the fungus imparts to the host improved tolerance to drought, insects, diseases, and nematodes as well as greater seedling vigor and growth potential (Latch, 1997).

The developmental basis of plant/endophyte mutualism is of considerable interest in plant biology due to the similarities and differences with the two best-studied plant-microbe interaction types, virulence/susceptibility and avirulence/resistance (Alfano and Collmer, 2004). Is the endophyte invisible to the host, treading so lightly as to go undetected? This seems unlikely given the observations that endophytes tend to be highly host-specific, and in rare instances when they can be transferred between host species they elicit a variety of defense-like responses in these non-native hosts even though those same grasses naturally harbor related endophytes (Christensen, 1997; Eaton et al. 2010; Koga et al. 1993).

Signal transduction pathways are key in interactions between plants and fungi. Such pathways continue to be studied intensively in several plant-associated fungi, including the causal agents of rice blast (*Magnaporthe grisea*), corn smut (*Ustilago maydis*) and chestnut blight (*Cryphonectria parasitica*), and toxigenic *Aspergillus* species. Regulation is mediated by expression or activation of numerous transcription factors, which then can induce or inhibit expression of other genes involved in interactions with the host (Basse and Farfing, 2006; Degani et al. 2004; Hoffmeister and Keller, 2007; Nathues et al. 2004). Similarly, signal transduction pathways and transcription regulation associated with host responses to pathogens are under intense investigation, with ongoing characterizations of pathways signaled by salicylic acid, jasmonic acid, ethylene, abscisic acid, nitric oxide and reactive oxygen species. Numerous transcription factors and metabolic mediators of these plant pathways have been identified (Dong et al. 2003; Fan and Dong, 2002; Kachroo et al. 2004; Kesarwani et al. 2007; Nandi et al. 2003). Recently, new transcriptomic tools are aiding in the dissection of the molecular interactions in mutualism between plants and rhizobia, mycorrhizal fungi and endophytes (Eaton et al. 2010; Høglund et al. 2009; Martin and Nehls, 2009).

The presence of the endophyte in tall fescue has consistently been shown to contribute benefits important in stand persistence and productivity (Clay, 1987). These benefits include: enhanced drought tolerance, increased tillering, improved root growth and above-ground biomass accumulation, enhanced ability to acquire mineral phosphate from soil, improved nitrogen utilization, and anti-nematode activity (Arachevaleta et al. 1989; Assuero et al. 2002; Elmi et al. 2000; Panaccione et al. 2006; Timper et al. 2005; West et al. 1993). However, the mechanisms by which these benefits occur remain uncharacterized. Further, current findings have yet to clarify how tall fescue and endophyte communicate to enable adaptation to each other.

Besides osmotic adjustment, research in plant drought tolerance has focused on other physiological changes. Even the root architecture is altered in endophyte-infected versus endophyte-free tall fescue in a manner that is reminiscent of an auxin effect, and likely to increase water acquisition (Malinowski et al. 1999). Other physiological changes that may occur are those that protect from reactive oxygen species [ROS] (Delauney and Verma; Tanaka et al. 2006). The endophyte possibly helps fight drought, in part, by reducing the associated oxidative stress in various ways, such as producing ROS-scavenging metabolites or up-regulating host genes encoding biosynthetic enzymes for antioxidants like resveratrol (Powell et al. 1994).

Transcriptome analysis presents a powerful approach to evaluating the dynamics of forage grass responses to environmental variations, and to formulating hypotheses for mechanisms of enhanced resistance to biotic and abiotic stresses, as well as forage interactions with their endophytes. Available genomic resources for the *Lolium*/*Festuca* complex and their endophytes have been meager, although these are expanding (Dinkins et al. 2010; Hesse et al. 2007; Mian et al. 2008; Voisey et al. 2007). Given the scarcity and need for these resources, we have elected to sequence cDNA libraries from tall fescue and meadow fescue, that has been used to begin to provide a starting point for molecular dissection of the tall fescue/endophyte association, as well as providing tools in subsequent proteomic and metabolomic analysis (Dinkins et al. 2012). Understanding the forms of metabolic cross-talk between plant and endophyte should allow more refined control of endophyte benefits and detriments during abiotic stress conditions.

## Experimental Design:

Genetically identical tall fescue (cv. KY31) clone pairs harboring the common toxic endophyte were isolated using the fungicide tebuconazole [(RS)-1-p-chlorophenyl-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol] in order to access the effects of the endophyte in the same genetic background (Dinkins, unpublished). The stock plants and fungicide-treated clones were examined for the presence or absence of endophyte by tissue print immunoblot (An et al. 1993) Nucleic Acid</keyword><keyword>Serotyping</keyword><keyword>Symbiosis</keyword></

keywords><dates><year>1993</year><pub-dates><date>May</date></pub-dates></dates><isbn>0099-2240 (Print, PCR and microscopy and propagated for several clonal generations prior the initial experiments. Ramets consisting of three tillers of similar size were planted into 8.5 x 8.5 cm square pots in sand, in the greenhouse. Sand was chosen as the growth medium because it allows even and rapid drying, and provides for easy harvesting of roots. Plants were watered twice daily for six weeks before subjecting them to experimental conditions to allow for regeneration and accumulation of sufficient biomass for sampling. After sufficient re-growth had occurred (3-4 weeks), water was withheld from the test group, while control plants were watered twice daily. Pots were randomized on the greenhouse bench, once while setting up the experiment and again before subjecting them to treatments, to minimize the bias caused by micro-environmental variation. Three pots of each clone pair were sampled as replicates from each treatment as endophyte infected watered controls (E+D-), endophyte infected water-deficit stressed (E+D+), endophyte free watered controls (E-D-), and endophyte free water-deficit stressed (E-D-), on each day from day 0 to day 5. Samples were harvested between 8:30 a.m to 9:30 a.m each day, collected samples immediately frozen in liquid nitrogen and subsequently prepared for RNA isolation as described below. The samples were divided into shoot, pseudostem, crown and root material. Five to six pots subjected to water-deficit conditions from each clone (E+/E-) were placed back into a daily watering regime on each harvest date until 5 days after withholding water in order to monitor their ability to recover from the drought stress. Live tiller numbers were counted after four weeks of recovery.

RNA was extracted using TRIzol® Reagent (Invitrogen Corporation, USA). The RNA was treated using TURBO DNA-free, (Ambion, Applied Biosystem, USA) for removal of contaminating DNA from RNA samples and for the removal of DNase after treatment. The integrity of RNA was checked by using the Bio-Rad Experion Automated Electrophoresis Station (Bio-Rad Laboratories, Hercules, CA, USA). The Illumina TruSeq RNA sample preparation kit (Illumina, Inc, San Diego, CA, USA) was used library preparation including adapters for pooling for sequencing analysis done at the Iowa State University DNA Facility. The first samples were sequenced using the Illumina Genome Analyzer II (GAII), and subsequent sequencing runs were done using the Illumina HiSeq2000. Two biological replicates for each treatment were run.

Sequence analysis was done using CLCbio Workbench. The reads were first mapped the *Neotyphodium coenophialum* contigs and *Epichloe festucae* genome (available at <http://www.endophyte.uky.edu>) to remove the fungal sequences. Reads per kilobase per million reads (RPKM) value was used to compare the relative hits on our in house *Lolium* assembly (Dinkins et al. 2012) and further filled in with the RNA-Seq reads. The new assembly comprised of 65,312 contigs (labeled as 65K Assembly) of which 36,912 matched (Blastx 1E 10-5) 12977 of the 37767 annotated *Arabidopsis thaliana* proteins (TAIR 10.0 – [www.arabidopsis.org](http://www.arabidopsis.org)), and 41,264 matched (Blastn 1E 10-5) 9975 of the 25219 annotated *Brachypodium distachyon* CDS gene models (Brachy 1.0 – [www.phytozome.net](http://www.phytozome.net)). An RPKM value of 20 was used as a cutoff for comparisons among the treatments.

## Results and Discussion

Clone 27 was randomly chosen from among a number of recovered clones following fungicide treatment for the experiment primarily due to its propensity to tiller rapidly in the greenhouse. While analysis of a single tall fescue clone is not indicative of the heterogeneity observed for tall fescue endophyte interactions in the field, it is akin to analysis of single cultivars in self-pollinating species. The analysis of E+ and E- plants of this clone (i.e. single genotype) allowed for comparisons of the endophyte effect within a single genotype. Overall the E+ plants survived the stress conditions imposed during the experiment better than the E- plants when number of tillers produced upon recovery was used as the measure. However, by day 5 days stress, none of the E+ and E- plants were able to recover (Figure 1). Day 2 was chosen for RNA-Seq analysis since differences in recovery was observed from the controls, i.e. plants exhibited stress, yet regrowth and recovery was observed, as well as differences observed in the recovery, based on the number of tillers that survived between the E+ and E- plants (Figure x). RNA expression levels was compared between endophyte-infected water controls (E+WC); and day 2 stressed (E+D2); endophyte-free water control (E-WC); and day 2 stressed (E-D2) in the pseudostem tissues as this is the region that contains the highest endophyte concentrations (Hinton and Bacon, 1985).

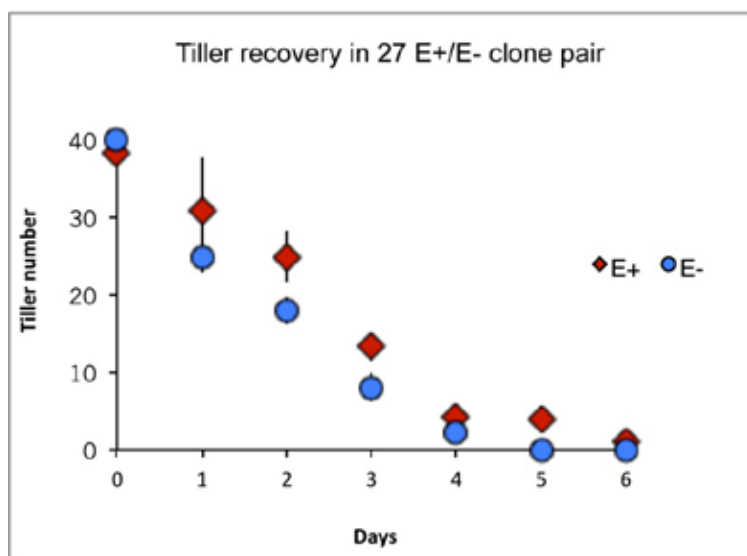


Figure 1. Number of tillers following stress treatments for tall fescue clone 27.

The first step in the analysis was to remove the fungal reads. As shown in Table 1 a high number of reads matched fungal sequences in the E+ plants, although a number of reads from the E- plants matching was also observed. Those matching the E- plant sequences were subsequently found to match primarily to fungal housekeeping genes that are expressed at high levels (data not shown). This was probably due to the presence of other fungi on, and in, the plants assayed since these plants were not grown axenically. The remainder of the RNA-Seq reads mapped onto the 65K assembly. The number that matched the tall fescue assembly is shown in Table 1. The majority of the reads that matched the 65K assembly, roughly 88% for all treatments, matched to single contigs, and those matching multiple contigs represented regions of similarity on closely related genes, or alternative transcripts from the same gene that assembled into different contigs.

Table 1. Illumina RNA-Seq reads matching fungal and tall fescue assembly sequences.

	Total Reads	% Match to Fungal <sup>1</sup>	% Match to Assembly <sup>2</sup>	% Unique
E- WC	51,219,944	0.31	69.48	87.67
E+ WC	53,496,201	2.76	71.57	87.42
E- D2	32,058,077	0.39	67.42	88.06
E+ D2	63,570,072	3.58	58.71	87.88

- 1 Percent match to *Epichloe fescucae* and *Neotyphodium coenophialum* sequence found at <http://www.endophyte.uky.edu>.
- 2 Percent match of the Illumina reads minus the fungal matches that matched to the 65K tall fescue assembly.
- 3 Percent of the reads that matched the 65K tall fescue assembly that mapped to a single contig.

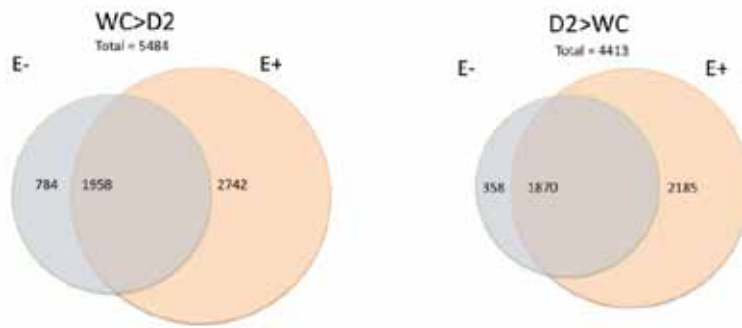


Figure 2. Number of differentially expressed contigs comparing water control and stress for endophyte-free (E-) and endophyte-infected (E+) plants.

In order to monitor differences in expression between water-control and stressed plants, as well as between the E+ and E- plants, a two-fold differences was used as a cut-off where a minimum RPKM value of 20 was observed for the highest expressing treatment. Overall, stress resulted in changes in expression in roughly 15% of the assembly contigs. This is similar to observed results in a number of species where stress has been observed to alter expression in 10-30% of the genome (Shinozaki and Yamaguchi-Shinozaki, 2007). Similar genes involved in stress responses, heat-shock related, ABA-responsive, documented in other species, were observed to be differentially expressed due to the stress treatment in our study. Although differential expression was found in both E+ and E- plants, both increased and decreased expression roughly equally, interestingly, more changes in expression were observed in the E+ plants than in the E- plants (Figure 2). A number of changes observed higher in the E+ plants, were called (i.e. > 2-fold) even though a similar trend was also observed in the E- plants, simply that 2-fold difference was not found. This can be seen in Figure 3 for the genes involved in proline biosynthesis that are expected to be expressed higher under stress (Delauney and Verma, 1993; Yoshida et al. 1997).

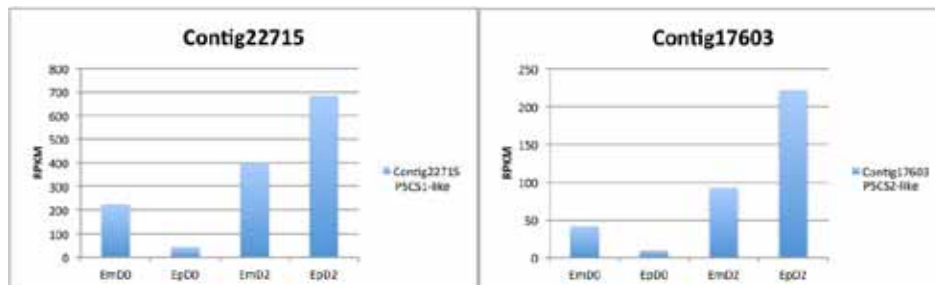


Figure 3. Comparison in expression of genes involved in proline biosynthesis, P5CS1-like and P5CS2-like contigs, for endophyte-free (Em) and endophyte-infected (Ep) under control and stress conditions.

Interestingly, the differential expression is due to two different causes; first, expression is higher in the E- than E+ under non-stressed conditions, whereas it was observed to be higher in the E+ plants under stress. The reason for the observed differences in expression under non-stressed conditions is not known as proline was barely above detectable levels, although proline content tended to be slightly higher in the E+ under the stress conditions (data not shown).

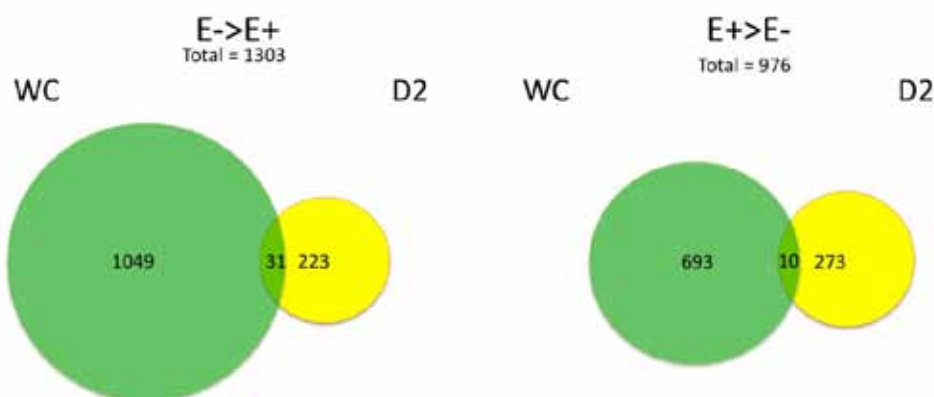


Figure 4. Number of differentially expressed endophyte-free (E-) and endophyte-infected (E+) tall fescue contigs.

Comparing expression between the E+ and E- plants roughly 2200 contigs were found to be differentially expressed (Figure 4). And where a significant number of genes were differentially expressed due to stress, the majority of the differentially expressed genes based on the presence/absence of the endophyte were found under the non-stressed treatments. Only 41 contigs were differentially expressed between the two irrespective of treatment; 31 that were expressed higher in the E- plants and 10 that were higher in the E+ plants. In the latter case, 9 of the 10 match genes homologous to glycosyl hydrolases and one did not have a significant match to any protein or gene in the NCBI database. Of the 31 that were expressed higher in the E- plants, 20 had significant matches known plant genes, a fourth corresponding to heat shock proteins. The specific functions of the differentially expressed proteins, including the putative glycosyl hydrolases described above, in conjunction with the presence/absence of the endophyte remains to be determined.

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## Development of a new genetic map for testing effects of creeping wildrye genes in basin wildrye backcross populations

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### Background

Perennial grasses display divergent adaptations in terms of growth habit, phenology, stress tolerance, seed dormancy, and many other functionally important traits. Basin wildrye [*Leymus cinereus* (Scribn. & Merr.) Á. Löve] is the largest native grass in western North America. It was once common on some of the most productive croplands of this region, such as Cache Valley region surrounding Utah State University, and it is still found on many sites where water and soil accumulate. However, like many native bunchgrasses of this region, it has a high growing point that is susceptible to grazing and clipping. Creeping wildrye [*Leymus triticoides* (Buckley) Pilg.] is strongly rhizomatous grass, which has good clipping tolerance, and was once thought to be dominant species in Central Valley of California, where it is still cultivated as a saline biomass crop. Interspecific creeping x basin wildrye hybrids display a combination of tall plant height from basin wildrye and strong rhizomes from creeping wildrye (Larson et al. 2006) that may provide good biomass yield potential and clipping tolerance.



Two mapping populations, TTC1 and TTC2, were previously derived from crosses of two *Leymus triticoides* × *Leymus cinereus* interspecific hybrids, TC1 and TC2, to one creeping wildrye recurrent parent (Wu et al. 2003; Larson et al. 2012). These creeping wildrye backcross populations were used to map QTLs controlling the inheritance of plant height, rhizomes, forage quality (Larson and Mayland, 2007; Larson et al. 2006). However, QTL analyses of the TTC mapping populations may not fully explain differences between species, especially for traits that may have dominant genes from creeping wildrye. Thus, a new full-sib genetic mapping population, which comprised of 250 genotypes was created from a backcross of the TC1 hybrid to basin wildrye. This population, designated TCC, was developed to test the effect of creeping wildrye genes in the basin wildrye background. This new *Leymus* genetic map is first described in this report.

## Construction of a new TCC genetic map of *Leymus*

A new TCC mapping family was developed from a backcross of the same TC1 hybrid to a basin wildrye recurrent parent. The TC hybrids and backcrosses were named after the species epithets of the *Leymus triticoides* (T) and *Leymus cinereus* (C). Molecular markers were developed from expressed gene sequence tags (ESTs) from rhizome and tiller meristems of the TC hybrids (Bushman et al. 2008; Kaur et al. 2008). Most of the 12,000 *Leymus* ESTs have been aligned to *Brachypodium* and other grass genome reference sequences on the Biofuel feedstock genomics resource from Michigan State University (<http://bfgr.plantbiology.msu.edu/>) and GrainGenes (Larson et al. 2012). The new TCC family was genotyped with 412 AFLP markers and 81 *Leymus* EST markers that were mapped into 14 linkage groups (LG) spanning 2389 cM (Table 1). By comparison, the current TTC consensus map (Larson et al. 2012) contains 375 AFLP framework markers and 376 *Leymus* EST markers in 14 LGs spanning 2381 cM (Table 1). The TTC and TCC maps were aligned using 46 shared AFLP and 48 shared EST marker (Table 1). Thus, a total of 435 *Leymus* EST markers mapped on the TTC and TCC map, including 28 marker loci for nine of the ten known lignin biosynthesis genes (Table 1.).

**Table 1.** Summary of TTC and TCC molecular marker maps

	TTC Consensus Map							TCC Map						Shared		Total
			Lignin		Cross-species	Map length (cM)	cM per locus			Lignin		Map length (cM)	cM per locus		EST (lignin)	<i>Leymus</i> EST
	AFLP	SSR	STS	STS				Total	AFLP	SSR	STS					
LG1a	28	18	1	4	51	135.9	2.7	28	2	1	31	110.4	3.6	2	2(1)	21
LG1b*	43	20	0	1	64	166.9	2.6	21	3	0	24	110.8	4.6	1	2	21
LG2a	39	18	2	4	63	188.5	3	21	8	0	29	217.4	7.5	2	3	25
LG2b	23	34	1	4	62	207.2	3.3	28	6	0	34	147.9	4.4	2	2	40
LG3a	31	28	2	6	67	200.8	3	30	10	0	40	221.6	5.5	4	9	33
LG3b	28	20	0	5	53	164.7	3.2	28	4	0	32	225.8	7.1	2	4	20
LG4Ns	16	29	0	5	50	168.1	3.4	24	4	0	28	149.2	5.3	5	2	31
LG4Xm	18	25	0	7	50	127.3	2.5	33	3	0	36	173.6	4.8	3	1	27
LG5Ns	27	27	3	3	60	194.8	3.2	41	2	0	43	155.3	3.6	6	1	34
LG5Xm	25	27	3	4	59	160.5	2.7	22	1	2	25	167.3	6.7	4	2(2)	34
LG6a	26	22	1	1	50	179.2	3.6	27	9	1	37	164.5	4.4	3	5	29
LG6b*	26	18	2	1	47	153.6	3.3	40	14	0	54	164.7	3.1	5	7	29
LG7a	23	33	4	2	62	171.7	2.8	36	2	3	41	184.1	4.5	5	4(2)	42
LG7b	22	31	7	1	61	161.8	2.7	33	6	2	41	196.1	4.8	2	4(2)	49
Overall	375	350	26	48	799	2381	3.0	412	74	9	495	2389	4.8	46	48(7)	435

## Comparison of QTL effects between reciprocal TTC and TCC backcrosses

QTL differences between the reciprocal backcrosses can be explained by non-additive gene effects. Genes that are dominant in the recurrent parent or recessive in the donor parent will not show phenotypic segregation and are not detectable by QTL analysis. Conversely, genes that are recessive in the recurrent parent or dominant in the donor parent will show phenotypic segregation and are detectable by QTL analysis. Additive-effect genes will show phenotypic segregation in reciprocal backcrosses and should be detectable by QTL analysis in either direction.

Comparisons of plant height QTLs between the TTC and TCC reciprocal backcrosses show that one QTL was present in both backcrosses, three QTLs were unique to the TTC *L. triticoides* backcross, and one QTL was unique to the TCC *L. cinereus* backcross (Fig. 2). *Leymus cinereus* contributed positive alleles for plant height QTLs on LG1a



in both TTC and TCC reciprocal backcrosses. Thus, we deduce that these plant height QTLs on LG1a are the result of the same additive effect gene (Fig. 2). The *L. cinereus* donor parent also contributed positive alleles for plant height QTLs on LG2a, LG3b, and LG4Xm in the TTC *L. triticoides* backcross, but no significant effects were detectable in the reciprocal TCC *L. cinereus* backcross (Fig. 2). Thus, we deduce that the *L. cinereus* LG2a, LG3b, and LG4Xm plant height QTL alleles are dominant (Fig. 2). Conversely, the *L. triticoides* donor parent contributed positive alleles for a plant height QTL on LG5Xm in the TCC *L. cinereus* backcross, but no significant effects were detectable in the reciprocal TTC *L. triticoides* backcross (Fig. 2). Thus, we deduce that the *L. triticoides* LG5Xm plant height QTL allele is also dominant (Fig. 2).

Comparisons of rhizome QTLs between the TTC and TCC reciprocal backcrosses show that one QTL was present in both backcrosses, two QTLs were unique to the TTC *L. triticoides* backcross, and one QTL was unique to the TCC *L. cinereus* backcross (Fig. 3). *Leymus triticoides* contributed positive alleles for all rhizome QTLs. Thus, we deduced that the *Leymus triticoides* LG2a rhizome QTL is dominant because it was detectable only in the TCC *L. cinereus* backcross (Fig. 3). The LG6a *L. triticoides* rhizome QTL had very strong effects in the TCC *L. cinereus* backcross and relatively weak, but significant effects in the TTC *L. triticoides* backcross (Fig. 3). Thus we deduce that the LG6a *L. triticoides* rhizome QTL has a strong, partially dominant effect (Fig. 3). Interestingly, *L. triticoides* rhizome QTLs on homoeologous regions of LG3a and LG3b were detectable only in the TTC *L. triticoides* backcross and not in the TCC *L. cinereus* backcross (Fig. 3). Thus, we deduce that *L. triticoides* rhizome QTLs detected on homoeologous regions of LG3a and LG3b both have recessive gene effects (Fig. 3).

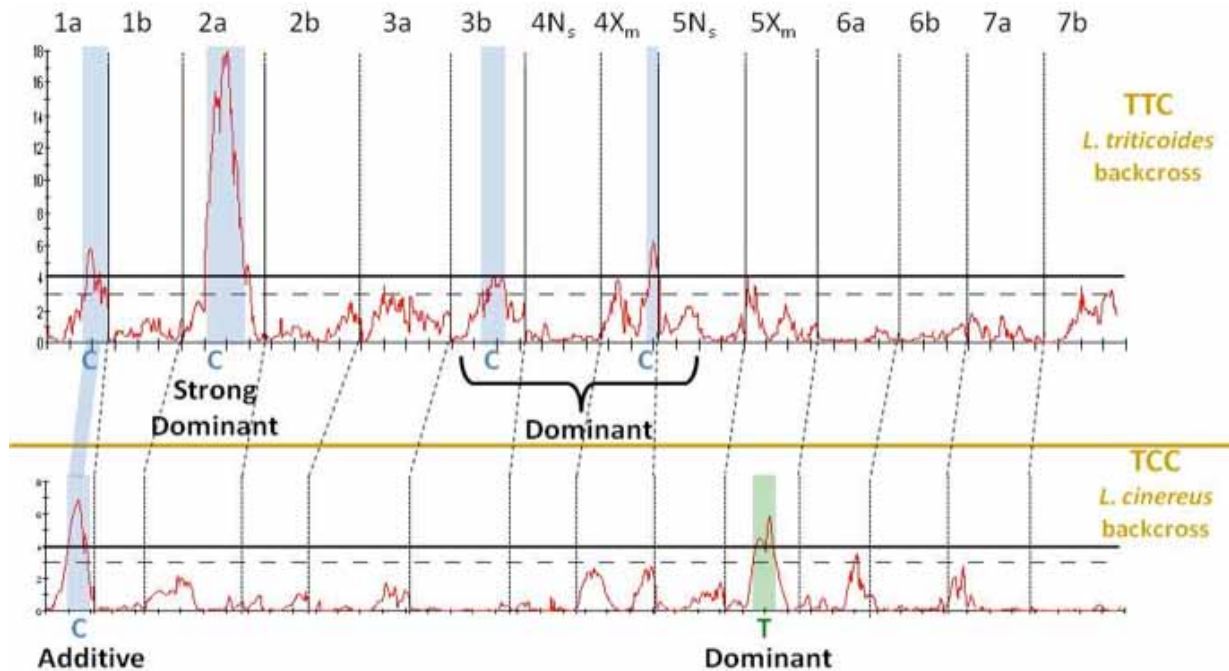


Figure 2. Comparison of genome-wide plant height QTL scans, over 14 linkage groups, between reciprocal TTC and TCC backcross families derived from *L. triticoides* (T) and *L. cinereus* (C) hybrids. Shaded boxes and letters indicate the positive-effect parent allele, with deduced gene effect annotations.

## Conclusion

Interspecific creeping x basin wildrye hybrids display a combination of dominant plant height genes from basin wildrye and dominant rhizome genes from creeping wildrye that may provide good biomass accumulation potential and clipping tolerance. Strong, major-effect genes with dominant or partially dominant effects were detectable for both plant height and rhizome traits. The relatively short creeping wildrye parent contributed one dominant plant height gene, which could theoretically provide plant height heterosis. The combination of additive and dominant plant height genes, from both parents, may explain the relatively tall plant height of the *L. triticoides* x *L. cinereus* hybrids, which equaled or exceeded the taller basin wildrye parent (Larson et al. 2006). Conversely, the combination of one

dominant, one partially dominant, and two recessive creeping wildrye rhizome genes explain why the rhizomatous spreading of the hybrids was intermediate between creeping wildrye and basin wildrye (Larson et al. 2006).

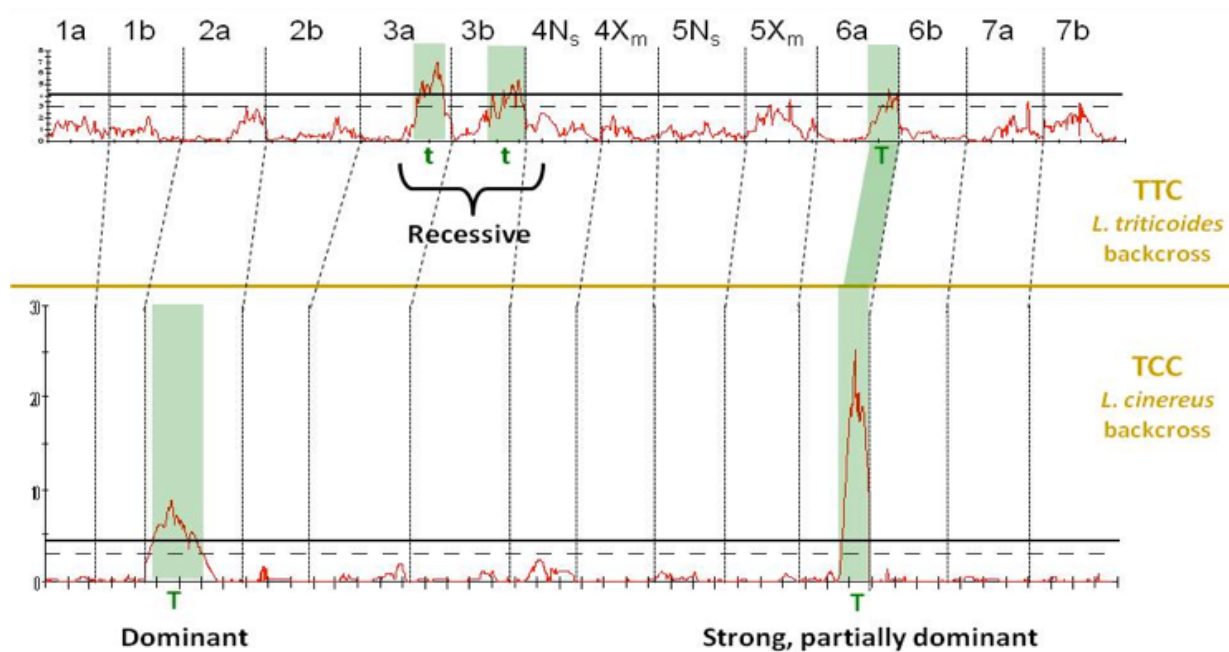


Figure 3. Comparison of genome-wide rhizome QTL scans, over 14 linkage groups, between reciprocal TTC and TCC backcross families derived from *L. triticoides* (T) and *L. cinereus* (C) hybrids. Shaded boxes and letters indicate the positive-effect parent allele, with deduced gene effect annotations.

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# Candidate Gene Association Mapping of Cold Hardiness in Perennial Ryegrass

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## Abstract

Association mapping provides a powerful tool for dissecting complex quantitative traits. Cold hardiness influences survival of perennial grasses in temperate regions. We conducted candidate gene association mapping of cold hardiness in 192 perennial ryegrass (*Lolium Perenne* L.) accessions. The population panel showed significant variations in phenotypic traits of percentage of winter recovery, chlorophyll index, and normalized difference vegetation index. Five population structures were found in the population assessed by 109 simple sequence repeat markers in perennial ryegrass. A total of 346 non-rare single nucleotide polymorphisms (SNPs) were discovered by sequencing 14 candidate genes involved in antioxidant metabolism, dehydration, and water movement across membrane and signal transduction. The model implementing population structure eliminated 90 % of false positive correlations, compared to simple linear model. Eleven significant associations between genes and traits were identified after controlling population structure. The results provide important information for further gene validation and genetic improvement for cold hardiness of perennial grasses.

## Introduction

Perennial plants growing in temperate zones survive harsh winters through cold acclimation, a phenomenon in which plants increase their freezing tolerance in response to decreasing day-length and low non-freezing temperatures, followed by subfreezing temperatures through fall and winter (Thomashow 1999; Kalberer et al. 2006). Cold hardiness is a complex process involving environmental signals, gene expression, physiological mechanisms, and empirical responses (Kalberer et al. 2006). Compared to the extensive research in cold acclimation in the model and economically important species, relatively little is known about the genetic mechanisms of cold acclimation in perennial grass species used for forage and turf.

Perennial ryegrass (*Lolium perenne* L.) is one of the most important forage and turf grasses in temperate climates zones in the world for its high quality for forage and landscaping as well as for other elements of ecosystem services such as carbon sequestration, soil formation and protection, and nutrient cycling. However, this grass species has demonstrated its inability to survive in a harsh winter. Perennial ryegrass is a self-incompatible diploid ( $2n = 2x = 14$ ) out-crossing species (Cornish et al. 1979) that shows a high degree of genetic diversity within the population (Kubik et al. 2001; Xing et al. 2007). There are more genetic and genomic resources available in perennial ryegrass (Jensen et al. 2005; Gill et al. 2006; King et al. 2008) than for any other major perennial grass species with more complex genomes. Thus, perennial ryegrass provides a good model for studying the genetic basis of cold hardiness. The results obtained from this species can be transformed into an investigation of other perennial grass species with more complex genomes.

Association mapping is a powerful high-resolution mapping tool for complex quantitative traits (Yu et al. 2006; Zhu et al. 2008; Ersoz et al. 2009; Brachi et al. 2010). Through exploitation of historical recombination events at the population level, association mapping studies link genes with traits, offer fine mapping, and test large numbers of alleles (Yu and Buckler 2006). Using candidate gene association mapping, two SNPs in *ScCbf15* and one in *ScCbf12*, leading to amino acid exchanges, were significantly associated with freezing tolerance in rye (Li et al. 2011). Furthermore, genome-wide association has proved to be a more powerful tool for genetic dissection of complex traits in model plants of *Arabidopsis thaliana* (Atwell et al. 2010; Brachi et al. 2010; Chan et al. 2011) and in crop species (Yan et al. 2010; Zhao et al. 2011). Several statistical tools have been developed to deal with false positive correlations caused by population structure and relative kinship when conducting association mapping (Yu et al. 2006; Zhang et al. 2010). To date, the candidate gene-association mapping of flowering time has been reported in perennial grass (Sköt et al. 2007; Sköt et al. 2011), but association mapping of cold hardiness have not been reported in perennial grass species. Therefore, the objective of this study was to identify candidate genes associated with cold hardiness in perennial ryegrass.

## Materials and Methods

The perennial ryegrass mapping population consisting of 192 accessions was collected from 43 counties, representing wild, cultivated, and undefined materials with wide geographical diversity according to USDA classification. All these accessions are diploids verified using flow cytometry technique (Wang et al. 2009a). Each accession was propagated by tillers and the population was established in the field in West Lafayette, Indiana and Wanatah, Indiana in the fall of 2008. We followed recommended practices for all routine inputs and managements. Assessment of cold hardiness was conducted in March and April in 2009 and 2010.

Whole-plant measurements were taken to indicate plant cold hardiness, including visual rating of canopy green coverage (CGC, %), normalized difference vegetation index (NDVI) and canopy chlorophyll index. Based on our observations, leaves of some genotypes turned green and recovered from the winter while leaves of some genotypes were still yellow at this time when samples were taken. Canopy green coverage was rated visually on a scale of 0 to 100, with 0 indicating completely dead or no green tissue coverage and 100 representing complete canopy coverage by green tissue. Data on NDVI was collected using Crop Circle (Holland Scientific Inc., Lincoln, NE, USA), obtained from calculation of  $(\text{reflectance at near infrared wavelength} - \text{reflectance at red wavelength}) / (\text{reflectance at near infrared wavelength} + \text{reflectance at red wavelength})$ . Canopy chlorophyll was measured using FieldScout CM 1000 Chlorophyll Meter (Spectrum Technologies, Plainfield, IL, USA).

The population of perennial ryegrasses was screened by using 109 simple sequence repeat (SSR) markers developed in perennial ryegrass (Jensen et al. 2005; Gill et al. 2006; King et al. 2008). Population structure was examined using SSR marker data with STRUCTURE software 2.3.1 (Pritchard et al. 2000). Fourteen candidate genes were selected for sequencing, including 10 putative genes encoding antioxidant enzymes (CAT, MnSOD, FeSOD, chloroplastic Cu/ZnSOD, cytosolic Cu/ZnSOD, APX, GPX, GR, DHAR, and MDHAR) and additional putative genes of *LEA*, *PIP*, *TIP*, and *MRAK*. Candidate gene association analysis was performed using the GLM by implementing Q model and simple linear model with the TASSEL 2.1 software package (Bradbury et al. 2007). The experiment was randomized complete block design with three replications for each accession in each location. Each genotype for all replications across locations was genetically identical. Phenotypic data for each accession from two locations were combined for association analyses.

## Trait Variation

Large differences in traits of cold hardiness were found in the mapping population of perennial ryegrass (Table 1). Among accessions, the minimum and maximum values were 0 and 79% for canopy green cover (CGC), 62 and 212 for canopy chlorophyll index (Chl), and 0.15 and 0.67 normalized difference vegetation index (NDVI), respectively; across the experimental locations. The cold tolerant accessions had higher CGC, Chl, and NDVI than that of the sensitive accessions. The results indicated that accessions varied in their capacity for cold hardiness, which provides an important basis for association analyses of genes with traits.

Table 1 Variation in percentage of recovery (PR), canopy chlorophyll index (Chl), canopy normalized difference vegetation index (NDVI) for perennial ryegrass accessions.

Trait	Minimum	Maximum	Mean	Std <sup>a</sup>
CGC	0	79	31.5	18.0
Chl	62	212	100.4	24.0
NDVI	0.15	0.67	0.33	0.07

<sup>a</sup> Standard deviation

## Population Structures

The STRUCTURE analysis identified five groups (G1, G2, G3, G4, and G5) in this panel of perennial ryegrass (Fig. 1) based on likelihood plots of the models and stability of grouping patterns across different runs (Wang et al., 2009b). The mean major allele frequency, gene diversity, and observed heterozygosity level ranged from 0.36 to 0.40, 0.71 to 0.76, and 0.42 to 0.53 across the five groups, respectively. The group G1 was the largest and most diverse group, with 121 accessions of mixed origins, including all the accessions from Oceania and the majority of the accessions from the U.S., Canada, Europe, and South America. Approximately 89.4 % of cultivated materials were assigned into G1

including 10 out of 11 turfgrass cultivars. The group G2 contained 21 accessions; from Europe (14), Asia (6), and one turf-type commercial cultivar from USA. The group G3 was the second largest group, with 25 accessions; mainly from Europe (13), Africa (7), and Asia (3). The group G4 contained 13 accessions, mainly from North Africa (Algeria, Morocco, and Tunisia). The group G5 was the smallest with 12 accessions mainly from Southern Europe and Asia. There was no obvious kinship in this population (data not shown).

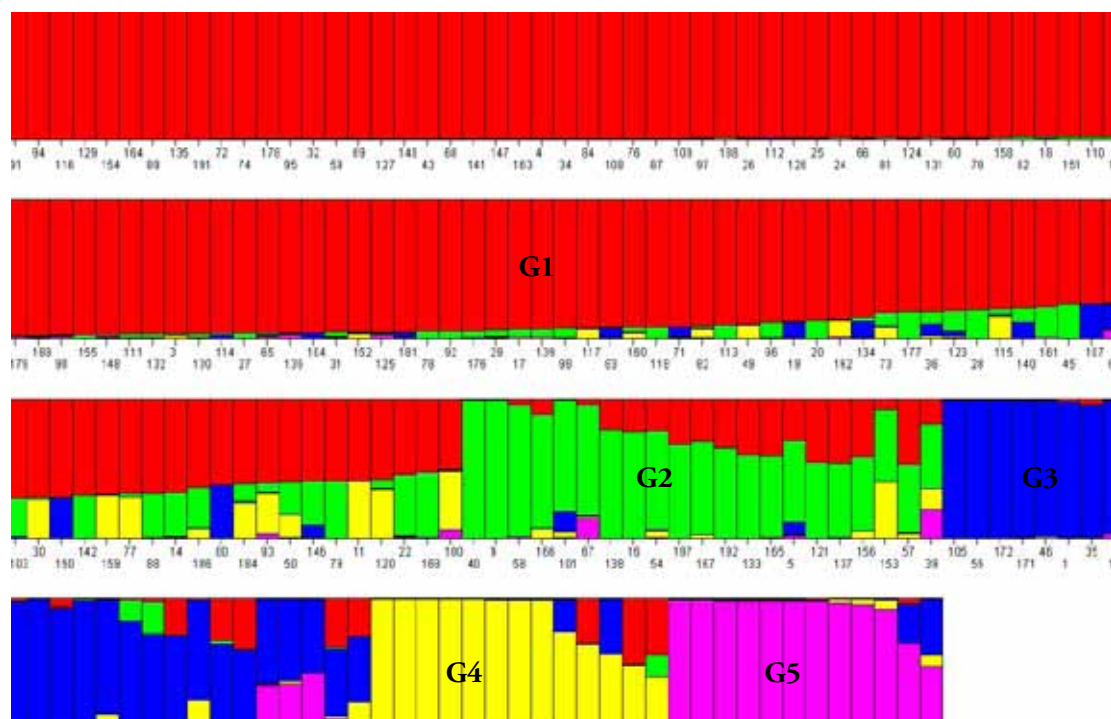


Figure 1. Genetic relatedness of 192 perennial ryegrass accessions with 109 SSRs as analyzed by the STRUCTURE program. Numbers on the y-axis indicate the membership coefficient. The color of the bar indicates the five groups identified through the Structure program (G1 = red, G2 = green, G3 = blue, G4 = yellow, and G5 = pink). Accessions with the same color belong to the same group.

## Association Analyses of Selected Genes

A total of 346 non-rare single nucleotide polymorphisms (SNPs) were discovered by sequencing 14 candidate genes involved (data not shown). Significant association between candidate genes and traits were found in the population of perennial ryegrass after controlling population structure (Table 1). Specifically, *MnSOD* was associated with NDVI at locus 51; *CAT* was associated with CGC and Chl at locus 494; *LpLEA3* was associated with CGC and Chl at locus 219 and 251, respectively. Association was also found between *Chl Cu/ZnSOD* and NDVI at locus 370 in the mapping population.

Table 2 Association of candidate gene with phenotypic trait of normalized difference vegetation index (NDVI), percentage of recovery (PR), and chlorophyll content (Chl)

Trait	Candidate gene	Locus	P-value
NDVI	<i>MnSOD</i>	51	4.21E-04
CGC	<i>CAT</i>	494	9.07E-05
Chl	<i>CAT</i>	494	1.58E-04
CGC	<i>LpLEA3</i>	219	1.95E-04
Chl	<i>LpLEA3</i>	254	2.66E-04
NDVI	<i>Chl Cu/Zn SOD</i>	370	4.73E-06

## Conclusion

The diverse accessions of perennial ryegrasses showed significant variations in phenotypic traits of canopy green cover, chlorophyll index, and normalized difference vegetation index in the early spring at the experiment sites. Five population structures were found in the population. Significant associations between genes and traits were identified after controlling population structure. Further research is needed to validate gene-trait association, and this will benefit genetic improvement for cold hardiness of perennial grasses.

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# Molecular Marker Identified Selfed Progeny and their Breeding Implications in Tetraploid Alfalfa Synthetics

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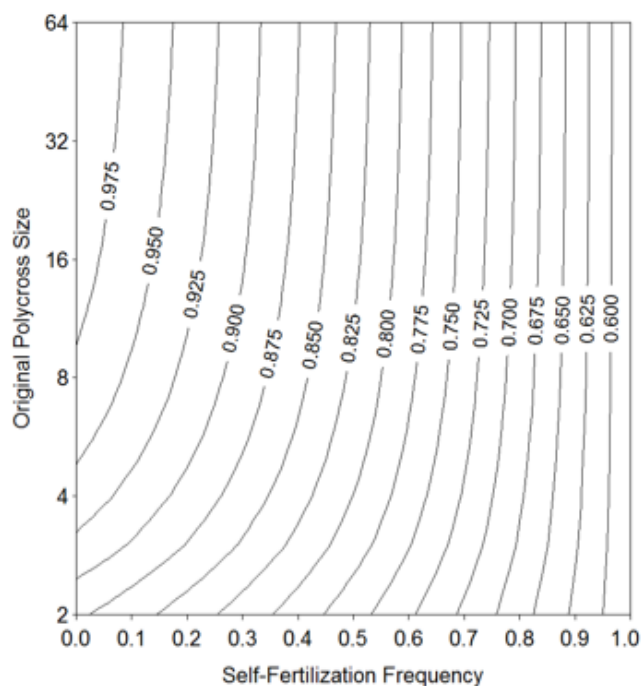
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## Abstract

Alfalfa (*Medicago sativa* L.) is a major forage legume. Selfing (i.e., self-pollination) in alfalfa is possible, particularly in the absence of pollen from another genotype. Selfing has also been shown to occur in insect pollinated seed production fields. Reported selfing rates under field conditions range from 2% to 53%. By and large alfalfa breeders have ignored selfing in their plant breeding programs except in breeding scheme requiring intentional selfing. Even modest selfing rates will significantly increase inbreeding depression, particularly in successive synthetic seed increase generations. Inbreeding depression due to selfing dwarfs inbreeding effects associated with broad versus narrow based synthetics. Prior to molecular markers, identifying selfed progeny in a standard alfalfa polycross or seed production field was almost impossible. In this study we present a SAS software program and simple DNA test utilizing SSR markers amplified in one or two PCR reactions that can identify selfed progeny in tetraploid polycrosses. The DNA test is demonstrated on a 15 parent autotetraploid alfalfa polycross pollinated with leafcutter bees (*Megachile rotundata* F.). In the demonstration polycross a 45% selfing rate was observed, consistent with previous studies. Outcross and self progeny were phenotyped for biomass yield and the synthetic's genetic load was estimated. Based on the synthetic's genetic load and selfing rate we estimated a 7% reduction in biomass yield from the syn 1 to syn 3 generation. One way to mitigate this yield loss would be to actively select for reduced selfing. A large range in selfing rates were observed between genotypes suggesting that selection for this trait should be feasible.



**Figure 1.** Expected inbreeding (contours =  $1 - F$ ) of syn 3 seed (certified) depending on original polycross (syn 0) size and self-fertilization frequency in an autotetraploid synthetic.

presented by Busbice (1969) it becomes clear that selfing during seed increase generations has a much larger theoretical impact on inbreeding if the synthetic is narrow based than if it is broad based (Fig. 1).

Prior to molecular markers, identifying selfed progeny in a standard alfalfa polycross or seed production field whose

## Introduction

Autotetraploid alfalfa *Medicago sativa* L.) ( $2n = 4x = 32$ ) is the third largest crop by acreage in the United States with an estimated 28 million kg of seed produced per year (NASS, 2009). Although alfalfa is generally considered to be an outcrossing species, selfing is possible, particularly in the absence of pollen from another genotype (Viands et al., 1988). Selfing has also been shown to occur in insect pollinated seed production fields (Burkart, 1937; Johansen, 1963; Bradner and Frakes, 1964; Pedersen, 1968; and Brown and Bingham, 1991). Reported field-wide selfing rates range from 9% to 53% with an average field selfing rate at 34%. Most of these earlier studies used white flowered “sentinel” plants to estimate field selfing rates. Brown and Bingham (1991), however, used purple flowered “sentinel” plants in a 0.2 ha modern variety seed production field pollinated with alfalfa leafcutter bees (*Megachile rotundata* F.), observing a 33% selfing rate. By and large alfalfa breeders have ignored selfing in their plant breeding programs except in breeding scheme requiring intentional selfing. In spite of this there has been a traditionally focus in alfalfa breeding research on narrow vs. broad based synthetics and the inbreeding implications of these two strategies (Busbice, 1969). Using models



plants did not contain a phenotypic marker was almost impossible. However, recently Riday (2011; 2012; 2013) has suggested using DNA markers to identify unknown parentage of progeny in forage breeding. Similar techniques could be used to identify selfed progeny (i.e., progeny resulting from self-fertilization). This study reports on the selfing rate in a syn 1 breeding polycross. Selfed progeny in this study were identified using an exclusion analysis (Jones and Ardren, 2003) based SAS software code (SAS, 2008) specifically designed to utilize codominant autotetraploid DNA markers with ambiguous dosage.

## Materials and Methods

A 15 parent polycross was established Apr 28, 2009 at the Cal/West Seeds Woodland Research Station in Woodland, CA (38°37'04.82" N, 121°47'55.35" W) in a Capay silt clay (fine, montmorillonitic, thermic Typic Chromoxererts) using elite breeding material internal to their program. The 180 plant polycross (6 x 30 plant rectangle) was set up with 15 parents clonally replicated 12 times. Two replications were included, each consisting of six-clone rows of each parent with plants spaced 38 cm apart and rows of different parental genotypes spaced 51 cm apart. Rows of plants consisted of six clonally replicated space-plants (of one of the 15 parents) spaced 38 cm apart. The 15 six-clone rows for the 15 parents were spaced 51 cm apart. A second set of 15 six-clone rows of the 15 parents were included as well.

Parent	Selfing Rate (%)	Seed Yield (g plant <sup>-1</sup> )
E09-006	13%	1.4
E09-014	33%	0.8
E09-020	57%	2.8
E09-027	68%	1.8
E09-032	13%	0.3
E09-035	79%	3.4
E09-036	51%	2.0
E09-039	48%	0.3
E09-040	15%	0.8
E09-041	69%	2.2
E09-042	50%	0.4
E09-043	70%	1.7
E09-057	47%	1.8
E09-079	20%	1.3
E09-085	48%	1.5
Average	45%	1.5
LSD (P < 0.05)	16%	---

**Table 1.** Selfing rate (%) and seed yield (g plant<sup>-1</sup>) of 15 alfalfa polycross parents pollinated with leafcutter bees during summer 2009 in Woodland, CA.

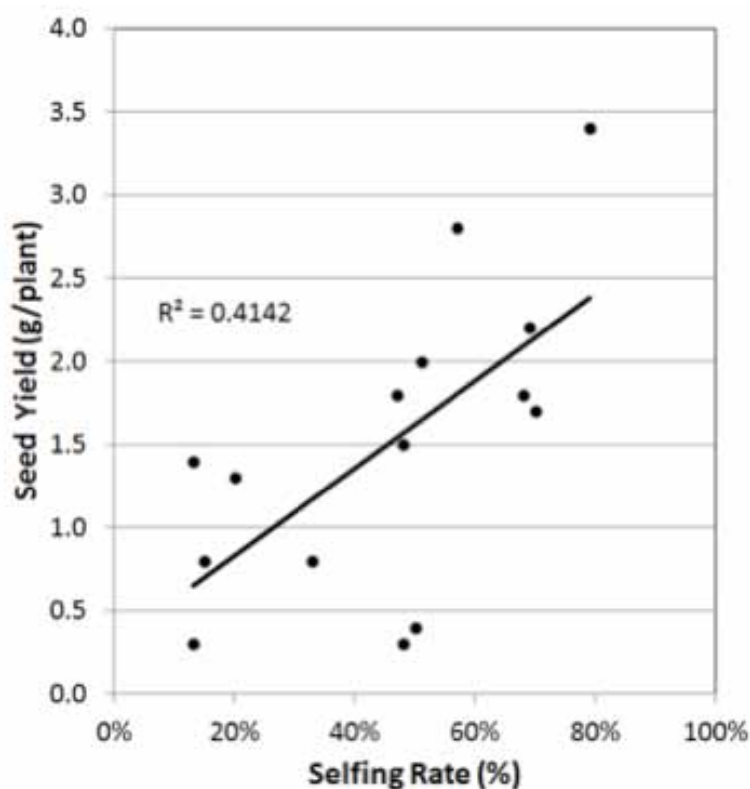
Plants were sprinkle irrigated until Jun 22 and furrow irrigated once on Jul 18. The polycross cage was sealed and a first set of alfalfa leafcutter bees was applied into the cage on Jul 13; a second set of bees was applied on Jul 24, totaling approximately 5000 bees. Plants were pollinated for 28 days. Upon seed ripening, bulked seed from each parent was harvested.

Equal numbers of progeny from the 15 parents that produced seed were space-planted into a breeding nursery in spring 2010 at the Cal/West Seeds West Salem, WI Research Station. Progeny plants from the 15 parent polycross were transplanted into 5-plant maternal-halfsib rows with plants spaced 45cm apart with 75cm spacing on all sides around the 5-plant plot. Individual plants were visually evaluated for vigor on: Jul 21, Aug 5, and Oct 8 2010; and Apr 25, Jun 3, Jul 12, Jul 28, Aug 29 2011. Vigor scores on each observation date were range transformed [ $100\% \times (\text{Score} - \text{Minimum Score}) / (\text{Maximum Score} - \text{Minimum Score})$ ]. Scores ranged from 0% to 100% with a 0% score indicating a dead plant. Repeated transformed scores of each plant were averaged to come up with an average vigor score (%) for each plant. Persistence (%) was also measured on Aug 29 2011 with living plants receiving a 100% score and dead plant receiving a 0% score.

Tissue from progeny plants in the West Salem breeding nursery was collected and DNA extracted (Riday and Krohn, 2010). Nineteen PCR amplified SSR markers (Sledge et al., 2005) (AFca11, AW146, AW235, BE119, BE323955, BF85, BG249, BG280, B28, AL45, AW86, BG230, Mt1G05, BI54, AW379, BG275, AW01, BE78, and BF220), were run on a 4-dye ABI Prism 3130xl genetic analyzer (Applied Biosystems Inc., Foster City, CA).

## Results and Discussion

A wide range in selfing rates was observed among the parents (13% to 79%) with an average rate of 45%. This rate falls within the observed insect mediated selfing rate observed in studies using sentinel plants to determine selfing rates (Table 1). Somewhat surprisingly parental seed yield was positively correlated with the parental selfing rate (Fig. 2). Strickler and Vinson (2000) note in reviewing previous literature that increasing flower number on plants has been associated with increased selfing, as pollinators are more likely to move between flowers on the same plant (i.e., more flowers per plant ~ more seed). Seed yield is an important trait in alfalfa breeding programs. If this correlation between seed yield and selfing rate has a genetic basis and is observed broadly in alfalfa populations, this may mean that alfalfa



**Table 2.** Average vigor rating (% of Maximum), Aug 2011 persistence, and inbreeding coefficient (F) of outcross and selfed progeny of 15 alfalfa parent polycross evaluated in West Salem, WI during 2010 and 2011.

breeding programs are selecting plants that have higher selfing rates via indirect selection for increased seed yields (i.e., and indirectly more flowers per plant). The self-fertility of these plants intrinsically may not be increasing but rather the morphology of the plant is causing pollinators to stay longer on the same plant.

An obvious question related to the observed selfing rate is what impact this rate has on varietal performance. The selfing impact would depend on the population's genetic load. One thought among alfalfa breeders is that over 100 years of alfalfa breeding has reduced the genetic load in alfalfa populations by increasing the frequency of favorable alleles and that elite alfalfa is more tolerant of inbreeding. Progeny from the polycross in this study were transplanted to the field. The mean vigor and 2<sup>nd</sup> year persistence of the two classes of plants were calculated (Table 2). Due to the DNA based paternity test, outcross versus self identity of field space-plants was known. The inbreeding coefficient of the self plants was estimated (selfs F = 0.17, assuming parents F = 0). Outcross progeny had on average a 64% average vigor score rating and were 100% persistence compared to 38% average vigor score rating and 83% persistence for selfs.

Using simple linear models proposed by Busbice

and Gurgis (1976) it is clear that the population would be extinct before it reached an inbreeding coefficient of one. This implies that the expected performance loss due to inbreeding is expected to be equal to F (Fig 1.).

In this study clonally replicated parents were placed in six-plant rows in the crossing blocks. Manipulating plant positioning in the syn 1 crossing block may reduce the selfing rate. Other cultural practices might also allow manipulation of the selfing rate (e.g., pollinator species, pollinator density). In syn 2 and syn 3 seed production fields (certified and foundation) options for cultural practice manipulation of the system may be more limited. If alfalfa breeders actively selected for self-incompatibility in breeding programs inbreeding associated with selfing could be eliminated. Genetic variation for the trait does appear to be present (Table 1). Some breeders would argue that selfs are more likely to be outcompeted during establishment. This may be true in hay fields; however, in seed production fields seeding rates are much lower than in hay fields. In addition there is a movement towards precision planting of seed in seed production fields where individual seeds are placed 10cm apart. Under such conditions selfs are much more likely to survive in seed production fields and contribute to inbreeding during seed increase.

This study was conducted on one breeding polycross. Further studies are required to look at alfalfa selfing rates under differing pollination conditions in syn 1, syn 2, and syn 3 seed production situations. Narrow sense heritabilities and genetic variation in a broader sampling of elite alfalfa germplasm should be examined to determine feasibility of selecting for self-incompatibility in alfalfa and to determine widespread presence or not of negative genetic correlations between seed yield and selfing rates. DNA markers allow inexpensive phenotyping of selfing rates in alfalfa requiring only maternal and progeny tissue and in most cases genotyping can be accomplished using only one PCR reaction.

	Avg. Vigor Rating (% of Maximum)	Aug 2011 Persistence (% Alive)	Inbreeding (F)
Outcross Progeny	64%	100%	0.00
Selfed Progeny	38%	83%	0.17

**Table 2.** Average vigor rating (% of Maximum), Aug 2011 persistence, and inbreeding coefficient (F) of outcross and selfed progeny of 15 alfalfa parent polycross evaluated in West Salem, WI during 2010 and 2011.

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## Next-Generation Solutions for Genomics-Assisted Breeding of Outbreeding Forage Plant Species

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## Abstract

Temperate outcrossing forage grasses and legumes have been the subject of increased resolution of genetic and genomic analysis for over a decade, supporting strategies for molecular breeding improvement. Nonetheless, relatively little progress has been made in terms of implementation in commercially relevant breeding programs. Factors such as the complex heterogeneous nature of varieties, limited knowledge of the breeding value of individual genotypes, non-extensive linkage disequilibrium and rudimentary phenotypic assessment procedures have all contributed to this shortfall. Progressive advances in genomics technology and rapidly developing expansion of phenomics capability are now capable of redressing this situation, in concert with a transition to a pedigree-based breeding system. A field-based nursery of clonally replicated plants from current elite varieties of both perennial ryegrass and Italian ryegrass has been established to test current tools for genotypic and phenotypic evaluation, supporting informed decisions for intermating selected individuals, and generating critical data on genetic relationships within

and between varieties. Genotypic methods have been based on large-scale discovery and verification of single nucleotide polymorphisms (SNPs), which is being extended to validation of efficient low-cost genotyping-by-sequencing (GBS) systems. Phenotypic measurements for key components of traits such as herbage quality have been automated for high-throughput delivery. A scheme for exploitation of these tools to enable genomic selection (GS) for ryegrasses is described, along with predictions of likely improvements of genetic gain and logistical efficiency. Further improvements may be obtained through radical re-design of genomic-assisted breeding systems, such as by manipulation of gametophytic self-incompatibility (SI) to drive hybrid generation. The current status of activities aimed at isolation and characterisation of the SI genes of perennial ryegrass is described, including physical mapping, transcriptome profiling and transgenic modification of candidate genes.

## Background

Over the last twenty years, there has been significant incremental development of genomic resources (including expressed sequence tags [ESTs] and genome survey sequences) and derived molecular genetic markers to support genomics-assisted breeding for temperate outcrossing forage crops, including grass and pasture legume species (Forster et al. 2008). This has now extended to advances in whole genome sequencing, especially for alfalfa and perennial ryegrass. Trait-dissection studies have used multiple marker systems, mainly based on biparental genetic mapping populations, and more recently, candidate gene-based association studies have been performed (Skøt et al. 2007). These processes have required the increased use of detailed phenotypic measurements, and have led to a broader exploration of germplasm diversity. However, despite this progress, there has been very limited real-world implementation in breeding programs delivering varieties to market.

This short-fall is due to a number of constraints, including the nature of breeding system, as cultivated varieties are based on polycrosses between multiple individuals, leading to high levels of genetic heterogeneity; a limited knowledge of the breeding value of individual genotypes, given that commercial breeding is usually based on re-sampling from existing populations or varieties, leading to an absence of pedigree data on an individual basis; and phenotypic assessment has been largely based on visual assessment of yield and persistence, with no real tradition of biochemical or physiological characterisation (due to cost and logistical considerations). In addition, the relationship between performance of individual parental plants in spaced-plant setting and synthetic progeny in swards is complex and requires further study.

Further constraints arise due to the magnitudes of effect of marker-tagged QTLs. These estimates have been largely derived from biparental mapping populations, and are generally small, unless for disease resistance and phenology traits. A meta-analysis from 23 published studies involving 560 ryegrass QTLs has revealed mean and median values close to c. 10% of phenotypic variance (H. Shinozuka, unpublished data). However, the well-known 'Beavis effect' demonstrates that these values are likely to be over-estimated, as suggested by genome-wide association studies of quantitative traits in rice (Huang et al. 2010) and maize. The experience in animal genetics is that individual loci typically account for very small components of measurable characters.

The final major constraint is due to limited extent of linkage disequilibrium (LD), which has been empirically demonstrated in most population types of perennial ryegrass, and certainly those involving diverse accessions (Ponting et al. 2007; Auzanneau et al. 2007; Xing et al. 2007; Fiil et al. 2011). Low LD arises due to the effects of large ancestral effective population size ( $N_e$ ) and allogamous reproductive habit, and restricts the capacity to identify enduring diagnostic relationships between marker alleles and trait gene alleles. In practice, this will limit the ability to reliably translate knowledge of marker – QTL associations from discovery to experimental populations, and across germplasm pools.

As a consequence, a core set of technologies and methods are required to address these constraints. Firstly, systems for intensive evaluation of individual plant genotypes, in order to allow a transition to a pedigree-based breeding system; systems for accurate, high-throughput phenotypic measurements of morphological, physiological and biochemical attributes; systems for accurate, high-throughput genome-wide sequence polymorphism detection, moving from current highly multiplexed SNP assays to future GBS methods; methods for experimental design, data management, analysis and prediction, including the value of simulation studies; and novel breeding technologies, including the capacity to select for host-microbe symbiota, and exploitation of hybrid vigour through control of SI. All of these components will provide an integrated system for implementation of GS in forages.

## Pedigree-based breeding system and high-throughput phenotyping

In the first category, a field-based nursery has been established and maintained for the last several years at DPI-Hamilton in western Victoria, Australia, containing perennial ryegrass and Italian ryegrass germplasm selected in concert with an industry partner, New Zealand Agriseeds (NZA). The design includes 1,000 genotypes from each species, with clonal replication, across a range of commercial and pre-commercial germplasm. The individual spaced plants have been subjected to intensive phenotypic characterisation for both morphological and biochemical traits. SNP genotyping at moderate multiplex ratio, through assessment of genetic relationships, supports selection of elite genotypes and targeted crosses for evaluation as mini-swards. The process can then be iterated through successive generations. The trial was established with clonal replicates as spaced plants that were assessed for herbage yield at three key time-points through the year, and in addition, all plants were assessed for herbage quality in late winter and early spring (Fig. 1). Scoring was also performed at the relevant time points for a number of other morphogenetic and disease resistance characters.

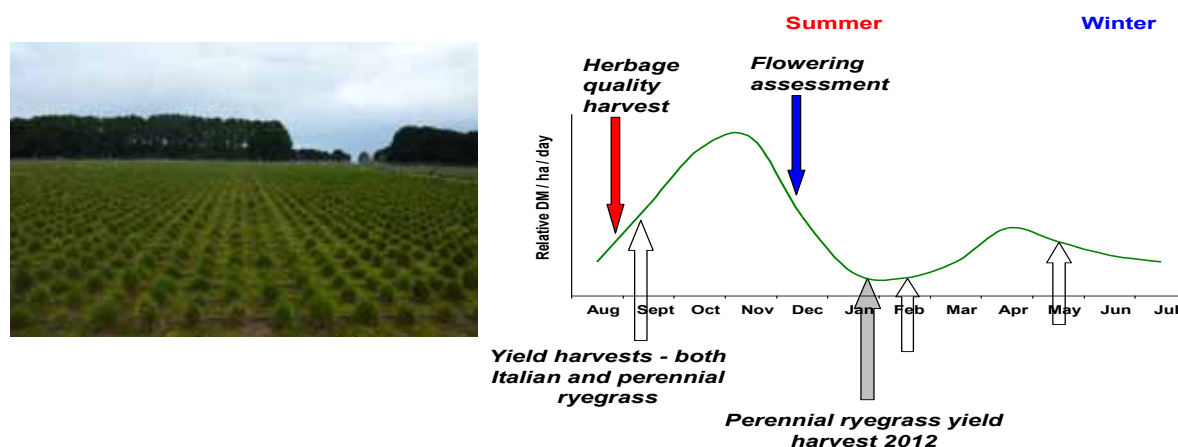


Figure 1. Establishment of the field-based nursery with clonal replication at DPI-Hamilton, and details of the herbage sampling strategy.

Detailed measurements of yield and nutrient content have been analysed on a cultivar-specific basis in terms of mean and distribution, demonstrating that cultivar identity exerts significant effects on measured traits within species, but significant variation arises between genotypes, with both moderate and extreme outlier genotypes. There is potential to develop a breeding value index for specific genotypes through weighting of each trait. For instance, a genotype with very high water soluble carbohydrate (WSC) content also exhibited very low biomass, hence compromising its breeding value. Heritability values over various ranges were determined, and were consistent with previous data from various studies. This data was used to select parents for the first-cycle of targeted crosses, and clearly provides information suitable for the design of genomic selection strategies.

In order to perform large-scale screening of plants for carbohydrate content, methods such as near infra-red reflectance spectroscopy (NIRS), high performance liquid chromatography (HPLC) are subject to constraints of cost and/or logistics. For this reason, a high-throughput quantification protocol was developed and implemented, and correlation was confirmed with HPLC-derived results. Method automation using liquid handling robotics delivered 11-fold increase in sample throughput, with values obtained for glucose, fructose, sucrose and fructan concentrations. The system is now being integrated with plant protein extraction and parallel quantification.

## Genome-wide sequence polymorphism detection

The genotyping tools that have been used in the clonal nursery experiment are derived from previous perennial ryegrass SNP discovery activities based on templates such as candidate genes (Cogan et al. 2006), low-resolution whole genome distributed loci, and complexity-reduced representations, collectively delivering c. 20,000 predicted SNP loci. An optimised sub-set with genome-wide distribution was formatted as a 384-plex Illumina GoldenGate™ assay, to provide a 'master genotyping tool' for genetic relationships and population structure assessment. A proportion of the

SNPs proved informative in Italian ryegrass, and the tool is capable discriminating between different ryegrass taxa, and between diploid and tetraploid varieties. The assays have also been transferred to an amplicon-based GBS system with significant reduction in cost. Application of the tool has also generated a comprehensive catalogue of cultivar affinities and differentiation, providing support for establishment of plant breeder's rights (Fig. 2).

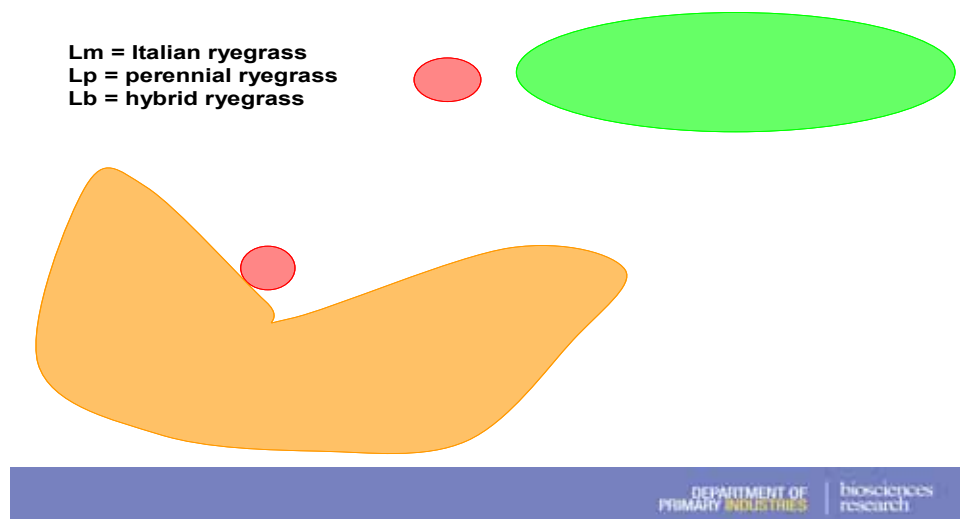


Figure 2. Schematic depiction of relationships between Italian ryegrass, perennial ryegrass and hybrid ryegrass varieties revealed through use of the 384-plex SNP genotyping assay.

For the next-phase of genomics tool development, the aim is to achieve whole-exome coverage (in the limit, 'a SNP in every gene'). The first stage has been to create a transcriptome atlas from a single genotype of perennial ryegrass (Impact<sub>04</sub>), based on sampling of multiple vegetative tissues, followed by deep-sequencing and annotation based on matching to the *Brachypodium distachyon* gene complement. The atlas is being extended, such as to reproductive tissues and the unigene set provides a reference template for SNP identification by resequencing. The same plant genotype has been used for gene-space assembly from whole genome sequence derived from Illumina platforms such as the HiSeq2000, with an estimated 70 X coverage for each allele. Contigs containing coding and control elements from representative genes have been assembled. The value of this approach has been exemplified by full description of gene families, such as those encoding nucleotide binding site – leucine-rich repeat (NBS-LRR) domain-containing genes responsible for qualitative disease resistance. The perennial ryegrass gene-space sequence has also provided a framework for assembly of lower-resolution whole genome sequence from related species, selected from major clades of the *Lolium-Festuca* complex, and including both Italian ryegrass and meadow fescue as cultivated species. This data can be used to identify orthologous genes, and is relevant for genomic studies of related polyploid species such as tall fescue. It is legitimate to anticipate that full exploitation of the exome-based sequence assembly will ultimately be based on combination with contiguous genome assembly, probably driven by 'third-generation' sequencing technologies. In the interim, gene-space ordering is possible through empirical genetic mapping and by using comparative genomics to assemble a 'virtual' physical map based on macrosynteny with *B. distachyon*. At present, GBS systems are being tested, including targeted sequence capture, and also exome framework-based complexity-reduced representations.

### Implementation of GS-based breeding schemes

Having developed the key technology approaches, it is necessary to decide how to assemble some of these tools to address limitations in current commercial breeding practice. Such programs are characterised by a limited number of recombination-selection events that are exploited within the breeding cycle, contributing to low observed rates of genetic gain (estimated at c. 7% per decade for perennial grasses [Gout and Jones 2006]). In a proposed GS scheme,

a number of key initial requirements are evident, based on prior experience in livestock genetics. GS theory states that large reference populations are required to predict accurate genomic estimated breeding values (GEBVs) when effective population size ( $N_e$ ) is large. So, in order to implement a logistically feasible initial design, it is proposed that  $N_e$  is reduced (and hence LD increased) firstly through the use of existing cultivar sources (exploiting the effect of prior 'bottlenecks'), and secondly, through use of within-family information (derived from full- and half-sibs). The scheme also aims to reduce generation interval by direct introduction of genotypes from the first selection round into subsequent rounds. GBS is used at low levels of resolution, through detection of genome-wide SNP variation in parental plants, and subsequent lower-density genotyping with imputation in the progeny.

Building on the success of the current field-based experiment, the scheme initiates with a clonal nursery containing c. 1,000 candidate genotypes, which are phenotypically assessed for traits such as yield and quality. A 15% selection pressure is then applied and high-resolution GBS is performed. Selection of parental genotypes is performed based on both phenotypic and genotypic data, and a series of pair-crosses involving common (bigamous) and independent (monogamous) parents are established to produce 50 pairs of half-sib families. The resulting progeny are then phenotyped and genotyped (at lower density, due to known pedigree relationships) and GS prediction equations are derived. The process can then be reiterated based on GEBVs for parental replacement. In addition, synthetic population development can be 'spun-off' at any stage, and additional characterised germplasm (for instance, containing a valuable transgenic event) can be used to refresh the reference population.

The scope of such a scheme can be extended to include other factors. Other target species would include tall fescue, white clover (for which the major additional factor would be the influence of allopolyploidy on genotypic analysis), or alfalfa (for which autopolyploidy influences allelic dosage determination, accurate haplotype reconstruction and verification of marker-trait gene linkage). Direct selection of optimised association in plant-microbe symbiota, such as fungal endophytes of grasses and rhizobia of legumes is a highly attractive option, but requires efficient methods for mass inoculation. Agronomic transgenes are another value-adding component. For all of these options, simulation studies would be valuable in order to determine: optimum population sizes; the impact of different selection strategies; the influence of different trait architectures; and lastly, the effect of altered breeding systems, in which control of SI can play a key role.

## Novel breeding methods based on control of SI

The significance and formal genetics of gametophytic SI in grasses are well-understood, and in previous studies, comparative genetic and physical mapping has defined the minimal genomic intervals that contain the *S* and *Z* loci (Shinozuka et al. 2010). Knowledge of SI allele content is important in ryegrass breeding for a number of reasons, such as to predict likely effects on fertility when producing restricted-base synthetics (with minimal numbers of parents). Development of partially inbred lines could help to mitigate the population size problems in GS schema, and to enable novel breeding schemes to exploit hybrid vigour.  $F_1$  hybrid production has been shown to be able to deliver large heterotic benefits for production traits (Posselt 1993), but is not currently practical for large-scale commercial programs.

Based on genomic sequence analysis, highly polymorphic loci have been identified flanking the perennial ryegrass *S* and *Z* loci, and a number of computational tools have been developed to predict SI locus haplotypes based on marker allele content. Empirical testing of predictive power in progress, and the suitability of technology for other species such as tall fescue has been tested and validated. As previously described in species such as red clover, this data can be implemented to reduce within-line variation and maximise complementary heterozygosity in a  $F_1$  hybrid population, with potential to accelerate genetic gain.

More desirable still, is actual gene identification to permit development of diagnostic allele-specific markers, and ultimately transgenic suppression of SI gene function. In our gene isolation strategy, BAC clones anchored by closely linked markers have been sequenced, allowing physical contigs to be assembled for both the *S*- and *Z*-containing regions. Ordering has been based on comparative microsynteny with model grass species, allowing gene prediction and identification of both conserved and ryegrass-specific genes, comprising a 'long-list' of (c. 60) candidates. In parallel, transcriptomic analysis of male and female reproductive tissue types has identified differentially expressed genes that reside within the *S* and *Z* locus regions, as well as novel additional candidate genes for both loci. A 'short-list' of SI candidate genes, (c. 5 for each locus) has been assembled. For verification, cohorts of transgenic plants have been generated using RNAi cassettes and phenotypic evaluation of primary transgenic plants is underway.



## Conclusions

In order to realise the objectives described here, improvements in genotyping technology (such as third-generation systems including single molecule real time [SMRT] technologies and nanopore-based separation) are predictable and non-limiting. In contrast, improvements in phenotyping technology not so predictable and could be highly limiting. They include advances in glasshouse-based phenomics (Walter et al. 2012), the ability to extend to field-based phenomics, and understanding of the relationship between phenotypes of individual plants and populations in swards. The ‘numbers game’ for GS implementation is complex, especially when dealing with symbiota, and so detailed simulations will be critical for quantification in design of experiments.

## Acknowledgments

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## From Breeding to Molecular Breeding: A 40 Year Perspective

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In 1972, my first year as a plant breeding graduate student, keywords for breeding articles in the journal *Crop Science* that year, and common to any plant breeder, were heritability ( $h^2$ ), disease resistance, genetic trait, forage breeding, forage yield, and mutant. Forty years later, common key words in recent *Crop Science* volumes include MAS (marker-assisted selection), QTL (quantitative trait loci), LOD (logarithm of the odds), and SSR (simple sequence repeats); these are also same key words used in the papers at this symposium where even its title, *Molecular Breeding of Forage and Turf*, has a definite technology feel. However, to a practicing plant breeder and cultivar developer, the main question that has evolved during the past 40 years is how to economically and efficiently apply all technologies, but especially the genomic and transgenic biotechnologies, to the breeding process?

## Reminders

*“People need to be reminded more than they need to be instructed”.* Dr. Samuel Johnson, 1709-1784. This quote

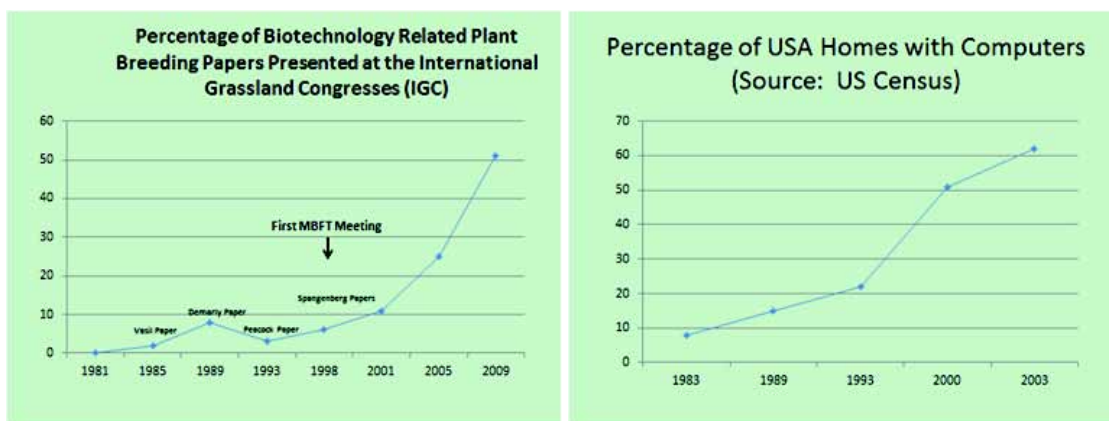
represents the underlying theme of this presentation. So, what would be some of things to remind people about that have happened in the past 40 years as well as future issues?

First, plant breeding is a profession with a proud and distinguished history. This is best shown by the classic 1929 picture of Professor R.A. Emerson, Cornell University, with his 4 students easily located through Google images (key words “Emerson, 1929, Ithaca”). Of these students, all proudly shown with their corn pollinating bags ready to go to the field, two of them, George Beadle and Barbara McClintock, later received Nobel Prizes in Physiology or Medicine. Likewise, and even more direct, was the 1970 Nobel Peace Prize awarded to Norman Borlaug, for his career as a plant breeder whose cultivars fed over a billion people in an effort called the Green Revolution. There have also been many other plant breeders, even forage and turf breeders such as Glenn Burton, who have had very productive careers when measured by their cultivars’ impact on agriculture and support the conclusion of a proud and distinguished history upon which we can all build a future.

Second, as with other science based disciplines, plant breeders have used technology to advance their efficiency. Even in my career, breeders have moved easily from harvesting forage yield plots with hand scythes to very sophisticated self-propelled harvesters with weigh cells and recording devices. Similarly, analyzing statistical data by hand using machines like the Monroe pinwheel calculator progressed into the computer age where now both personal computers and large computational “systems” make data storage and analysis much faster and definitely more efficient. Therefore, it was an easy step for most plant breeders to see the potential of genomics and transgenics as very sophisticated tools to further increase their efficiency. The importance of making this step is supported by a quote from Dr. Borlaug later in his career: “Producing food for 6.2 billion people, adding a population of 80 million more a year, is not simple. We better develop an ever improved science and technology, including the new biotechnology, to produce the food that’s needed for the world today” (see [http://en.wikiquote.org/wiki/Norman\\_Borlaug](http://en.wikiquote.org/wiki/Norman_Borlaug) ).

## Use of Biotechnologies

I had the pleasure of chairing the session on forage crop improvement during the 1993 International Grassland Congress (Bouton 1993). In those days, biotechnologies were already being touted, but very few of the offered papers at that Congress dealt directly with their use leading to the conclusion that their impact resided in their future potential and not their immediate use to plant breeders. However, even before 1993, the groundwork for using biotechnologies had already begun as seen in the accompanying figure which tracks the plenary papers presented at the same Congresses beginning in 1983 until the present. There are noteworthy findings in this figure: 1) from 1983 most papers were plenary or invited with the important ones presented by Drs. Vasil, Demarly, Peacock, and Spangenberg, and 2) the dramatic rise in papers coincided with the first MBFT meeting held in 1998. This lag phase in the use of new technologies, accompanied by a dramatic rise in their use, is not unlike adoption of any technology as seen by the adjacent figure that charts use of computers in US homes.



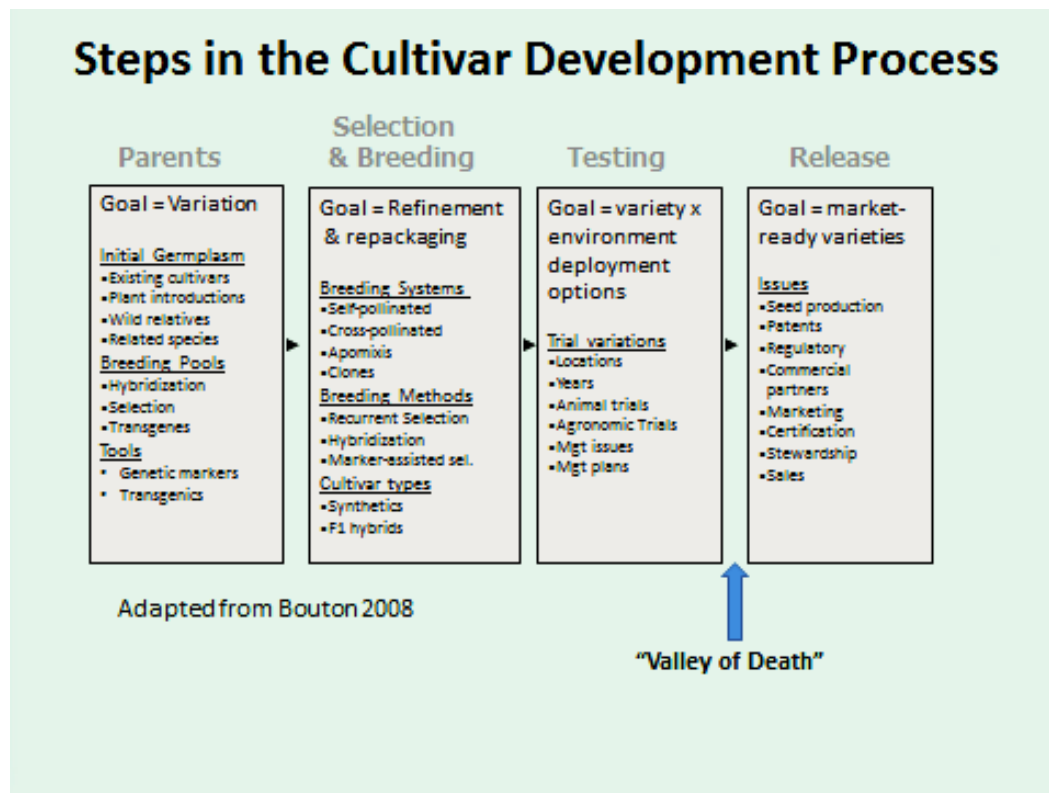
Therefore, genomic and transgenic biotechnologies are being routinely used in traditional forage and turf breeding programs with this area now described as molecular breeding (Bouton 2008).

## Issues

Today, the question is “when will biotechnology positively impact farmers”? Before that general question can be

answered one must address the specific question of “when will these biotechnologies be used routinely and extensively in forage crop cultivar development programs”?

The main issue for wide spread adoption and use is their cost and cost effectiveness for a breeding program’s main goals (Bouton 2012). Everything associated with the new technologies is expensive especially when compared to budgets of traditionally run programs. For one crop industries like corn, development costs and information are shared across many groups, but the forage and turf industries have many production systems and many more species that fit them making even the major species less investigated. Government investment has mitigated this cost somewhat, but it remains high and growing, and for many public and private breeding programs, too high. In fact, I, like many breeders, invested heavily into biotechnologies because of their potential to cost effectively speed up the selection and breeding phases allowing more of my resources to be spent on the final testing phase of the cultivar development process (see accompanying figure below) where most breeders will tell you is the most time and resource consuming step. So, my original plan to free up resources due to use of biotechnologies is now irrelevant and along with normal problems of cultivar performance in the various trials to prove their worth, it can create a “valley of death” for final release and commercialization of new cultivars (Bouton 2008). This also leads to a practical question: instead of going through the expense of incorporating complex persistence traits such as drought tolerance into drought insensitive species like perennial ryegrass, why not just have farmers use a drought tolerant species like alfalfa (Bouton 2012)?



“Non-science” issues are also now a real problem. For example, the oral presentation by Mark McCaslin at this symposium showed total costs estimates for transgene deployment to be over \$100 million USD. Regulatory costs are a big part of this cost and the regulatory process needs to be reformed to balance benefit and risk especially for perennial crops (Strauss et al. 2010). However, as pointed out by Dr. McCaslin, if a trait cannot command a high trait fee in addition to the proprietary seed price, and then realize a very high market share, it is not worth the risk to try and commercially deploy it. There are not many traits that have this type of market power; only traits that impart drought tolerance and higher nutritive quality, or remove pasture bloat would have that power. Another, non-science issue that has emerged for transgenics, which is especially pertinent for countries in Europe and Australasia, is the “green centric” view of its regulatory agencies and its citizenry. This is a depressing trend. At the same time, it is a tragedy, because transgenics is a powerful technology that needs to be fully utilized for the betterment of mankind and its agriculture.

So, the best advice to those who wish to continue to push for the practical use of transgenics is that you stay positive and adopt a missionary spirit: live in the moment, cherish your wins, forget your losses, and keep your eye on the final prize, but also increase efforts to educate a skeptical and uninformed public about their sustainability and safety (Bouton 2010).

Even for genomics, where the issues are less controversial, genotyping costs are dropping but phenotyping remains a significant problem (Bouton 2010). There are also practical problems, but these are mainly self-inflicted and therefore correctable. The underlying technologies such as genetic marker systems are always changing, requiring a frustrating increase in investment and a delay for their practical use. For example, my own alfalfa improvement program, with me as a willing participant, started with a genetic map based on RFLPs (20 years ago!) then migrated to an assessment of using RAPDs and then SSRs and now SNPs with no cultivar yet developed that has depended, even partially, on the use of this tool. This is changing for us, and we now use markers more routinely in the breeding program. However, the changing technology model may work well for the computer industry where the consumer is willing to change very quickly to a new technology, but in pastoral agriculture where 50 year old varieties are still used, this may not be economical or even practical. The turnover time for each generation of breeding is also long and when technologies continue to change it causes further delays in their practical use. Therefore, maybe it is time for someone, probably a plant breeder, to remind the genomicists where these tools fit in the overall cultivar development process (see accompanying figure). Or even better, say strongly “It is a tool, so find a way to cost effectively use it somewhere in the process”. Genomicists are clever people; they just needed to be “reminded more than they need to be instructed” that even the simplest ways to use genetic markers (e.g. structuring families, eliminating irrelevant plants before embarking on the expensive field tests, etc.) have potential for great impact!

Technology based resources are going to fewer and more traditional forage species because these crops are the ones that have greater economic value (Bouton 2007). Therefore, this will favor alfalfa, perennial ryegrass, and white clover as seen by the percentage of papers at this meeting centered on these crops. The concentration of resources into fewer species requires efforts on the part of those primary programs to find ways that technology developed for one species has utility across other species. To this end, web sites are now accessible that are building that important infrastructure in alfalfa that should have use in other legume crops ([www.alfalfagenome.org](http://www.alfalfagenome.org); <http://medsa.comparative-legumes.org>). **Finally, traits are important, but do not forget the base germplasm.**

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# Other Invited Oral Presentation Abstracts

## Determination of self-incompatibility genotypes in *Lolium perenne* through linked DNA marker genotyping

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Self-incompatibility (SI) is a mechanism under genetic control that prevents inbreeding by blocking fertilisation of ovules by like-pollen. Subject to frequency dependent selection, rare alleles, originating through mutation or ingression into populations, are at a selective advantage and theoretically increase in frequency under balancing selection. SI loci are therefore highly poly-allelic. The grass family shares a common two locus SI system controlled gametophytically by two loci, S and Z. Although the genes responsible for SI in grasses have not been identified yet, fine-mapping of S and Z in *Lolium perenne* has resulted in a number of close flanking markers. For this proof of principle study, we investigate the possibility of using S- and Z-linked marker variation to predict genotypes and their frequencies in plant populations. We used a population of 55 plants derived from a base population of nine genotypes that has been subjected to twelve generations of mixed and half-sib family selection. We screened non- and infrequently-recombinant S and Z DNA markers using high resolution melting curve analysis (HRM) which is highly discriminating, quick and relatively easy to perform. Marker genotypes were then grouped and relative S/Z genotypes were predicted. Marker polymorphisms were confirmed by DNA base sequencing. S/Z genotypes will be confirmed by making selected in-vitro cross-pollinations to demonstrate that the genotype combinations produce the predicted incompatible, half-, three-quarters- and fully-compatible pollinations. The ability to readily determine S/Z genotypes of grass plants will be of interest to population geneticists investigating properties of SI loci and their evolutionary significance. Being able to genotype large numbers of plants quickly and cheaply will also enhance breeders' ability to select plants on SI genotype in order to produce highly intra-incompatible, inter-compatible population combinations for the production of heterotic F<sub>1</sub> hybrid populations in forage, turf and bio-energy grass crops.

## Using a genotype by sequencing approach to estimate the extent of LD in a perennial ryegrass association population

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Knowledge on Linkage Disequilibrium (LD) is important when considering genotyping strategies for genomic selection. A rapid decay of LD necessitates the need for a higher marker density to enable markers to capture the entire phenotypic variation in the population. Outbreeding species with large effective population sizes are expected to have very low levels of LD. We have utilised a draft assembly of the perennial ryegrass genome to assess the extent of genome wide LD in a perennial ryegrass association population. The population consists of 172 single individuals originating from 72 varieties and 22 ecotypes, and there is a 92, 82 split between forage and turf types. The population was genotyped using a genotype by sequencing approach with the restriction enzyme APEKI (5bp cutter). Sequence tags were mapped onto scaffolds from the draft genome assembly that were greater than 10Kb in size, in order to identify SNPs with various minor allele frequencies. The genotypes were determined for each individual at these SNP sites, and used to calculate the squared correlation of allele frequencies between SNP pairs falling within the same genomic scaffold. This allowed us to estimate the rate of LD decay over distances up to 100Kb.

## The *Lolium* genome zipper – targeted use of grass genome resources for ryegrass genomics

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In the past decades, intense research efforts in model grass species has led to the establishment of whole genome sequences that constitute a major resource for genetic and genomic applications. In outbreeding forage and turf grass species such as ryegrasses (*Lolium* spp.), these resources are not available yet. Thus, targeted use of sequence resources from model grass genomes provides a major opportunity to efficiently exploit genomic information for genetic and breeding applications in non-model species.

## Characterisation of a Nodule Enhanced Malate Dehydrogenase Gene from White Clover (*Trifolium repens* L.)

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Aluminium (Al) toxicity is a major environmental limitation for plant production in acid soils which represent more than one third of the world's agricultural land. Most agricultural soils are deficient in phosphorus (P), and most soluble P, incorporated by addition of non-renewable P-fertilisers, is rapidly fixed into unavailable forms. Al-induced secretion of organic acids (OA) by roots, such as malate and citrate, has the capacity to chelate the toxic Al cation, excluding it from the root, which appears also associated with enhanced P-use efficiency. A white clover EST was identified as the putative orthologue of a *Medicago sativa* nodule enhanced malate dehydrogenase gene (MsneMDH). The EST sequence of the putative TrneMDH was then used to identify a white clover bacterial artificial chromosome (BAC) clone that would contain the complete gene. The identified BAC clone was subjected to sequencing using the GS FLX Titanium platform and assembled using Newbler v 1.0.52 software. A contig of c. 36 kbp was identified that contained the complete TrneMDH gene sequence as well as adjacent genes in both 5' and 3' directions. Extensive micro-synteny was identified and characterised between the white clover BAC sequence and the reference genome sequences of *Glycine max*, *Lotus japonicus* and to lesser extent *Arabidopsis thaliana*. The TrneMDH gene sequence was also subjected to in vitro SNP discovery and genetic mapping in an F<sub>1</sub> population, enabling its mapping to linkage group 8.

# Condensed Tannin Expression in Legumes

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Legumes are a diverse family, with many species contributing an important role in agricultural forage, by providing a valuable feed source while concurrently fixing atmospheric nitrogen to enhance plant growth. The presence or absence of condensed tannins (CT) varies greatly within forage and non-forage legumes. Condensed tannins are polymeric flavonoids, derived from the phenylpropanoid pathway, which play an important role in pastoral agricultural systems. Ruminants fed on forages containing moderate amounts of CT can improve a whole range of nutrition and production qualities for grazing livestock, including animal productivity, animal health and environmental aspects. However, the lack of CT can cause unique problems including bloat, inefficient protein utilisation and adverse loss of nitrogen (N) into the environment while high levels of CT in the diet of ruminants decrease palatability and feed intake. Sufficient foliar CT are present in some forage plants such as *Lotus corniculatus* (Birdsfoot trefoil) and *Onobrychis viciifolia* (Sainfoin), but such species often show poor persistence under grazing. In contrast, forage legumes that can persist in temperate grazing systems, such as *Trifolium repens* (white clover) and *Medicago sativa* (alfalfa), have only negligible amounts of CT in leaves. Since the quantities and chemical structures of such CT, as well as the spatial and temporal accumulation patterns, are extremely diverse, considerable efforts are currently directed towards understanding and modifying CT biosynthesis. CT biosynthesis and accumulation is still not fully characterized in legumes, but major advances have elucidated several steps in this complex pathway. Traditional breeding techniques attempts have increased the trace concentrations of CT present in some grazed legumes. In addition, new biotechnology approaches with transcription factors such as MtPAR and TaMYB14 aimed at increasing the levels of CT in important forage legumes, have yielded a number of promising opportunities. We will discuss these developments in this presentation.

## RNA-Seq transcriptome analysis of flowering time and vernalization response in perennial ryegrass

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The high forage quality provided during its vegetative growth phase makes perennial ryegrass (*Lolium perenne* L.) the predominant forage grass used in temperate agriculture. However, forage quality decreases significantly during the reproductive phase due to stem and inflorescence production. The controlled inhibition or delay of flowering would result in an extended period of vegetative growth and a significant increase of forage quality. Our goal is to develop and validate functional molecular markers suitable for marker-assisted breeding for delayed flowering time in perennial ryegrass. We used an RNA-Seq approach to investigate the transcriptome during primary induction (vernalization and short days) and secondary induction (warmer temperatures and long days), which are essential for initiation of flower induction in perennial ryegrass. We observed significant changes in the transcriptome related to different time points during primary and secondary induction. Candidate genes were selected based on expression profiles and work is ongoing to convert them into Single Nucleotide Polymorphism (SNP) molecular markers for vernalization and flowering response. The candidate genes and the SNP markers will be integrated in the perennial ryegrass genetic map.



# SNP discovery and candidate gene-based association mapping of forage quality traits in perennial ryegrass

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Repetitive DNA provides a challenge for performing targeted DNA capture in plants. An array based method and an insolution procedure (MYselect) were compared for optimal targeted DNA capture of 1300 genes in perennial ryegrass. The MYselect procedure gave superior results with >50% of reads on target. This protocol together with 454 sequencing is being used to identify SNPs and determine gene haplotypes from plants in elite breeding lines or diverse wild collections. Targeted DNA capture in combination with barcoding of samples will allow for a genotype-by-sequencing (GBS) approach. Several genes have been identified where loss-of-function gives an improved trait phenotype. Mutations potentially causing loss-of-function were identified by sequencing amplicons derived from DNA pools. Rapid decay of linkage disequilibrium in perennial ryegrass is well suited for candidate gene-based association studies to identify functional nucleotide polymorphisms (FNPs) responsible for trait variation. Nineteen candidate genes in the lignin and fructan metabolic pathways generally displayed rapid decay of linkage disequilibrium characterised by  $r^2$  values below 0.2 within distances of 1kb. A candidate gene in the fructan pathway displayed copy number variation. Association tests performed in a diverse association mapping population provided some evidence of correlation with fructan content but are inconclusive because of population structure.

## Genotypic and chemotypic diversity of epichloid endophytes

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Epichloid endophytes (*Epichloë* and *Neotyphodium* sp.) consist of both sexual (nonhybrid) and asexual (hybrid and nonhybrid) species that are fungal symbionts of many cool-season grasses. Most epichloae are capable of producing the bioactive anti-herbivore compounds, particularly ergot alkaloids, indole-diterpenes, lolines and peramine, and can exhibit considerable chemotypic diversity within these four alkaloid classes. In addition, somatic hybridization has the potential to provide increased herbivore protection for the host plant through pyramiding of the alkaloid biosynthesis genes. Molecular analyses of endophyte genetic traits from within and between host populations allow us to explore resident endophyte diversity present in single grass host species. PCR with genomic DNA extracted from individual plants (seeds or tillers) is used to rapidly predict alkaloid chemotypes at the EAS (ergot alkaloids), LOL (lolines), IDT (indole-diterpenes) and PER (peramine) loci as well as to generate sequence for phylogenetic inference of progenitor origins for these alkaloid genes. Analyses of housekeeping and mating type genes are used to solidify these phylogenetic relationships and verify hybrid versus nonhybrid status. Collections of *Elymus* spp. (*El. canadensis* and *El. virginicus*), *Bromus laevipes* and tall fescue have recently been evaluated and considerable endophyte chemotypic diversity has been identified within each host. Some hosts were able to harbor both hybrid and nonhybrid endophytes. Chemotypic diversity of the hybrids may have arisen from independent hybridization events and this alkaloid diversity likely translates into differences in fitness and persistence of the host.

## Multidisciplinary approaches to improve forage legume species for stressing environments in South America

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Traditional legume plant breeding develops in connection to detrimental plant-interacting organisms, selecting for well defined characteristics like partial or total resistance to pathogens or pests. But there are few examples of integrating plant and microbial selections for maximizing beneficial plant-microbe interactions, even the well known relationship with symbiotic nitrogen fixation microorganism. Participatory approaches are commonly interpreted as processes for farmers' plant breeding. Yet, integrated projects show that participatory research approaches can increase adoption of molecular and metabolic knowledge into applied plant breeding and improved plant and beneficial microorganism interactions. Symbiosis strongly relies on the environment and on both plant and microbial genetic diversity, as there is usually a certain degree of genotype-to-genotype specificity. In collaborative research involving plant and microorganism genetic resources the aim was to identify how best the partnerships might be facilitated to promote the newly arising cultivars. Plant breeding selection should be performed including the beneficial microbes in the target environment. Researchers involved in the projects LOTASSA and LESIS recognized the importance of symbiotic nitrogen fixation for the success of legume pastures in environmentally-constrained areas, screening existing plant and rhizobial resources in a way that the most reliable, best performing plant-microbe combinations in stressed South American soils were selected. Complex characteristics such as tolerance to abiotic stresses, such as soil salinity or acidity, were selected in plant and microbes in parallel, with final rounds to establish the most efficient plant-microbe combinations. Breeding without considering beneficial microorganisms may lead to progressive loss of the plant capacity to benefit from the microbes. Therefore, improved interactions and/or competitiveness with microbes involve simultaneous selection of most competent plant and microbial genotypes for the target environment. Improved knowledge of microbial interactions with new cultivars of *Lotus* and *Trifolium* may lead to improved and more reliable pastures in marginal soils of South America.

## Gene expression and metabolite analysis of endophyte infected and uninfected tall fescue clone pairs under water deficit conditions

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Tall fescue (*Lolium arundinaceum* L.) plants symbiotic with the endophytic fungus, *Neotyphodium coenophialum* (E+), have better survivability and persistence under stressful conditions, especially under drought stress, than plants lacking the endophyte (E-). To understand more about the grass-endophyte interactions and how endophyte affects the host plant physiology and gene expression, we conducted a time course water deficit stress experiment using genetically identical E+ and E- clone pairs of tall fescue. Upon re-watering, survival and retillering was significantly greater for E+ than E- plants starting from days 2 - 3 of the stress conditions. RNA-Seq analysis of pseudostem derived RNA comparing E+ and E- of Day 2 stress and control plants was done. CLCbio Genomic Workbench was used to generate contigs using the tall fescue cDNA sequences previously submitted to NCBI as a reference. The contig sequences were analyzed using Blastx and Blastn against the Arabidopsis thaliana, Brachypodium distachyon and rice genomes for comparison and putative annotation assignment. Only a few contigs/genes were differentially expressed between the E+ and E- plants under non-stressed conditions, but a large number were differentially expressed (>2X) between E+ and E- plants under the water deficit conditions. In addition, as expected, a large number of contigs/genes were differentially expressed due to the stress treatment. Metabolomic analyses revealed higher accumulation of the free sugars and proline in E+ plants at early days of onset of stress compared to E- plants. Loline alkaloids and mannitol, fungal metabolites, also increased with water stress. Analysis of specific genes is ongoing and results will be presented.

# Targeting the *Miscanthus* genome(s) for accelerated breeding of a novel energy crop

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In the face of an increasing population, limited land and water resources and a changing climate, we must ensure that sustainable land use solutions are developed and deployed to provide for our food, feed, fuel and fibre needs whilst maintaining biodiversity and other ecosystems services. *Miscanthus* is a C<sub>4</sub> grass native to much of South East Asia. It combines rapid annual growth with minimal inputs with tolerance to low temperatures and is therefore an ideal energy crop for temperate as well as tropical environments. Our research focuses on increasing *Miscanthus* yields in order to replace fossil fuel usage, sequester atmospheric carbon, and ultimately contribute to climate change mitigation, whilst minimising land take. Understanding of the genetic control of biomass performance traits is required in order to accelerate breeding efforts. A combination of germplasm characterisation, genetic mapping, transcriptomics and association studies are being deployed to develop trait-associated markers for use within the breeding programme at IBERS. Latterly, this genomic approach has been augmented with studies of the bacterial endophyte population present within *Miscanthus*. Initial work has sought to characterise the populations present and work is ongoing to determine whether the endophytes confer any advantage, such as nitrogen fixation or abiotic stress tolerance, to the *Miscanthus* host.

## Three ways to find a pool (or more): Comparison of DArT, SNP and SSR markers in elucidating heterotic pools in *Lolium perenne* (L.)

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Perennial ryegrass, *Lolium perenne* L., is the most important grass species in temperate regions. As an out-crossing species, it is highly heterogeneous and resistant against most biotic and abiotic stresses. Due to its good cutting tolerance and its high yield potential, *L. perenne* may serve as a sustainable source of biomass for energy production in biogas plants. In order to yet increase the biomass yield of perennial ryegrass via hybrid breeding utilizing heterosis, three types of molecular markers were compared in a project funded by the German FNR (Agency for Renewable Resources) for their ability to elucidate the presence of heterotic pools in *L. perenne*: 1384 DArTs, 182 SNPs and 51 SSRs were employed on 305 bulked genotype mixtures, the respective data being analyzed and results visualized with the help of the bioinformatic analysis tool DiSTo2 (Diversity Studies Toolkit 2). Based on these results and together with passport and phenotypic data, we expect to establish the basis for hybrid breeding for high biomass yields in *Lolium perenne*, facilitating the generation of new ryegrass varieties. Being an alternative to today's main crop for biogas production in Germany and world-wide, maize, this will eventually stabilize/increase the ryegrass production area and lead to an enhanced diversification of agriculture.

# Molecular breeding of *Miscanthus* spp., a potential bioenergy crop

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One of the most intensively investigated potential new energy crops is *Miscanthus* spp. that is native to eastern Asian countries such as Japan, China and Korea. Molecular breeding will offer good opportunities, especially for value added traits such as enhanced biomass, abiotic stress tolerance and fermentation efficiency. A molecular breeding program for *Miscanthus* to develop a novel energy crop has been started at the Hokkaido University, Japan. Recent research achievements of our group are following. Genetic transformation can be expected to be applied on *Miscanthus* to produce genetically modified plants with desired characters. Particle bombardment-mediated transformation has been established in *M. sinensis* (Wang et al. 2011). Successfully obtained transgenic *M. sinensis* plants with perennial ryegrass sucrose-sucrose 1-fructosyltransferase (prft4) accumulated fructan and showed less leaf wilting under low temperature. This possibly leads to higher biomass production in cold areas. Evaluation for genetic diversity using simple sequence repeat markers revealed that the accessions collected in Hokkaido Island was distinctly separated from those in other southern regions of Japan, which suggested that geographical proximity probably played a significant role in determining the extent of genetic diversity. Elucidation of genetic control of flowering time and content of lignin, cellulose, and hemicelluloses in cell walls is important for development of bioenergy crop. Homologues of flowering genes such as HD1 (CONSTANS) were isolated and characterized the polymorphism among accessions of *Miscanthus*. Also, lignin biosynthesis genes as phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT), 4-coumarate: coenzyme A ligase (4CL), cinnamoyl CoA reductase (CCR), and cinnamyl alcohol dehydrogenase (CAD) were isolated and transcript expression levels of these genes were analyzed in leaves and stem of the *Miscanthus* spp.. Molecular breeding in a conjunction with a traditional breeding program would develop novel bioenergy crop of *Miscanthus* for feedstock production.

## Linkage and Meiotic Analyses Suggest a Segmental Allopolyploid Origin of the Hexaploid *Brachiaria humidicola*

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Genetic linkage maps play fundamental roles in understanding genome structure, explaining genome formation events during evolution, and discovering the genetic bases of important traits. The African species *Brachiaria humidicola* (Rendle) Schweick is an important perennial forage grass throughout the tropics (Keller-Grein et al. 1996). It is a polyploid and apomictic species (Valle & Savidan 1996), which makes genetic studies more difficult, leading to limited genomic resources currently available. A F1 population derived from the cross between two heterozygous genotypes of the species, the sexual accession BRA005811-H031 and the apomictic cultivar *B. humidicola* cv. BRS Tupi, both  $2n=6x=36$ , was used for map construction and meiotic and apomixis analyses. The linkage map for the species was developed from 79 polymorphic SSR markers derived from the same species using the software OneMap based on the simplex (segregating 1:1) and double-simplex (segregating 3:1) markers. The map consisted of 38 linkage groups (LGs) and had a total length of 1,543.8 cM, with 63 microsatellite loci and an average map density of 12.3 cM. Two homology groups were formed with seven LGs while the other LGs remained ungrouped. The apo-locus was mapped in LG02, 31.3 cM from the mark/amplicon Bh026.e.D2. From bi- to hexavalents at diakinesis, two nucleoli in some meiocytes, smaller chromosomes with preferential allocation in the first metaphase plate, and asynchronous chromosome migration to the poles during anaphases were found in cytological analyses. This is the first linkage map of a *Brachiaria* species and this map will be useful for quantitative trait locus analysis in *B. humidicola* in the future, as well as genome evolution studies in *Brachiaria* species. The linkage map along with the meiocytes analyses confirm the previous reported evidences of hybridization (Boldrini et al 2010) and suggest a segmental allopolyploid origin of the hexaploid *B. humidicola*. Moreover, apomixis mapping is consistent with previous reports (Ebina et al 2005, Stein et al 2007) and confirms the necessity of further studies.

# Genomic and phenotypic instabilities in *Poa annua* L.

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*Poa annua* L. is one of the world's most widely distributed plant species and is ecologically and economically important both as a weed and as a forage and turfgrass; however, little information is available concerning the genetics of this species for breeding purposes. We used single copy nuclear DNA sequences *trx* and *CDO504* and chloroplast sequences *ndhF* and *trnTLF* to discern the evolutionary origin of *Poa annua* from all other possible origins. We show that at least two interspecific hybridizations between the maternal annual species *Poa infirma* and the paternal perennial species *Poa supina* gave rise to present-day *Poa annua*. Following this result, we were able to re-examine previously published cytological data from amphihaploids and present evidence for the genomic designations of *Poa infirma* as II and *Poa supina* as SS, making the genomic constitution of the allotetraploid *Poa annua* as IISS. As a species, *Poa annua* is most prevalent as an invasive, annual weed in ecosystems world-wide but there is also a dwarf perennial greens-type form that produces high turf quality with great utility to the golf industry. We characterized the morphological traits of the greens-type phenotype and investigated its inheritance and stability through genetic crosses. We found that the greens-type phenotype links to a single genetic mechanism. However, in advanced-generation progeny, the segregation of the greens-type phenotype does not conform to the disomic single-gene inheritance model. Additional models including tetrasomic inheritance, gene complementation, and quantitative inheritance models were also not applicable. These crossing results, along with the observation of somatic reversions, suggest that the greens-type phenotype is unstable and may be regulated by an epigenetic mechanism. Overall, our results place new emphasis on the extensive chromosomal rearrangements that took place during *Poa annua*'s evolutionary origin as the underlying cause of epigenetic modification of the greens-type phenotype.

## Molecular and Cytological Evaluation of Genetic Diversity in St. Augustinegrass

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St. Augustinegrass (*Stenotaphrum secundatum* (Walt.) Kuntze) is a widely used turfgrass species in warm, tropical and subtropical regions of the world. In the present study, flow cytometry, chromosome counts, and AFLP marker analysis were performed in order to assess genetic diversity at the molecular and cytological levels among public sources of *Stenotaphrum* germplasm. Cytological investigations identified five different ploidy levels (diploid, triploid, aneuploid, tetraploid, and hexaploid) with chromosome numbers ranging from  $2n=2x=18$  to  $2n=6x=54$ . Lack of agreement between flow cytometry-inferred ploidy level and chromosome counts was observed for higher ploidy genotypes. Therefore, DNA contents of these accessions are not simple multiples of the content of the diploid genome. This may indicate the existence of different genomes in the genus. Over 1700 loci were scanned with 15 AFLP primer pairs. All plant introductions and cultivars could be uniquely identified and the average pair-wise genetic similarity value was relatively low at 0.63. Results from both principal coordinate and cluster analyses showed well defined separation of accessions by ploidy levels. Moreover, results from the AMOVA indicated that 46% of the total variation could be explained by differences between ploidy levels. A clear positive correlation was observed between ploidy level and number of bands scored, with polyploids showing an increased number of bands. These results indicate that variation in chromosome number is an important source of genetic variation in St. Augustinegrass. Information derived from this study will aid in the strategic utilization of *Stenotaphrum* germplasm in cultivar development.

## Identifying SNP among diverse alfalfa genotypes using transcriptome sequencing

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Alfalfa, a perennial, outcrossing species, is a widely planted forage legume. Marker assisted breeding could enhance improvement of alfalfa, particularly for quantitatively inherited traits, if many genome-wide markers were available. The objective of this experiment was to sequence the transcriptomes of 27 alfalfa genotypes including elite breeding genotypes, parents of mapping populations, and unimproved wild genotypes, using an Illumina Genome Analyzer IIX in order to identify single nucleotide polymorphism (SNP). Between 17.2 and 31.6 million 72-bp reads were obtained for each of the 27 transcriptomes. De novo assembly of the reads generated 25,183 contigs with a total length of 26.8 Mbp and an average length of 1,065 bp, with an average read depth of 55.9-fold for each genotype. Overall, 21,954 (87.2%) of the 25,183 contigs represented 14,878 unique protein accessions. Gene ontology (GO) analysis suggested that a broad diversity of genes was represented in the resulting sequences. The realignment of individual reads to the contigs enabled the detection of 872,384 SNPs and 31,760 InDels. High resolution melting (HRM) analysis was used to validate 91% of 192 putative SNPs identified by sequencing. Both allelic variants at about 95% of SNP sites identified among the five wild, unimproved genotypes are still present in cultivated alfalfa, and all four US breeding programs also contain a high proportion of these SNPs. Thus, little evidence exists for loss of significant DNA sequence diversity due to either domestication or a century of selection. An alfalfa Illumina Infinium array with ~12,000 SNPs is being developed, which will enable high-throughput genotyping. The EST and SNP markers generated from this study are publicly available at the legume Information System (<http://medsa.comparative-legumes.org/>) and can contribute to future alfalfa research and breeding applications.

## Mechanisms of drought and salt tolerance in the fodder shrub *Zygophyllum xanthoxylum*

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*Zygophyllum xanthoxylum*, a succulent xerophyte with excellent adaptability to adverse arid environments and a fodder shrub with high palatability and nutrient value, colonizes arid areas in China and Mongolia. Here we reported the possible physiological and molecular mechanisms of drought and salt tolerance in this species. We found that *Z. xanthoxylum* increased growth responding to salt condition with a typical feature for halophytes and became more tolerant to drought in the presence of moderate salinity (50 mM NaCl). Our further experiments showed that NaCl at 50 mM mitigated deleterious impacts of drought on the growth of *Z. xanthoxylum* by improving the relative water content and increasing leaf turgor pressure due to a significant increase of the contribution of Na<sup>+</sup> to leaf osmotic potential and a significant drop in leaf water potential, and concomitantly, increasing chlorophyll concentrations and photosystem II activity resulting in an enhancement of overall plant photosynthetic activity. Subsequently, we found that two distinct low-affinity Na<sup>+</sup> uptake pathways existed in *Z. xanthoxylum*, one of which might be mediated by an AKT1-type channel; the plasma membrane Na<sup>+</sup>/H<sup>+</sup> antiporter ZxSOS1 involved in the transport and spatial distribution of Na<sup>+</sup> and K<sup>+</sup> and therefore regulated the growth of *Z. xanthoxylum* under salt condition; the tonoplast Na<sup>+</sup>/H<sup>+</sup> antiporter ZxNHX mediated leaf Na<sup>+</sup> accumulation by compartmentalizing Na<sup>+</sup> into vacuoles under salt and drought condition. Finally, co-expression of ZxNHX and vacuolar H<sup>+</sup>-pyrophosphatase gene ZxVP1 significantly improved both drought and salt tolerance in important legume forages *Medicago sativa* L. and *Lotus corniculatus* L. In summary, these findings suggest that *Z. xanthoxylum* is able to accumulate high concentration of Na<sup>+</sup> in its leaves and use it directly for osmotic adjustment under arid environments, which is coupled with an improvement in leaf hydration and photosynthetic activity; moreover, the transporters/channels involved in Na<sup>+</sup> and K<sup>+</sup> uptake, transport and vacuolar compartmentalization play vital roles for *Z. xanthoxylum* to adapt to both salt and drought conditions.

# Candidate Gene Association Mapping of Cold Hardiness in Perennial Ryegrass

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Association mapping provides a powerful tool for dissecting complex quantitative traits. Cold hardiness influences survival of perennial grasses in temperate regions. We conducted candidate gene association mapping of cold hardiness in 192 perennial ryegrass (*Lolium Perenne* L.) accessions. The population panel showed significant variations in phenotypic traits of survival rate, chlorophyll index, and normalized difference vegetation index. Five population structures and minimum relative kinship were found in the population assessed by 109 simple sequence repeat markers in perennial ryegrass. A total of 346 non-rare single nucleotide polymorphisms (SNPs) were discovered by sequencing 14 candidate genes involved in antioxidant metabolism, dehydration, and water movement across membrane and signal transduction. The model implementing population structure eliminated 73 % of false positive correlations, compared to simple linear model. Sixty significant associations between genes and traits were identified after controlling population structure. The results provide important information for further gene validation and genetic improvement for cold hardiness of perennial grasses.

## A recurrent selection approach for the identification of dehydrin variants linked to superior freezing tolerance in alfalfa

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The analysis of candidate gene polymorphisms is a powerful strategy to unravel the genetic bases of phenotypic variation of complex traits. Dehydrins belong to a complex family of intrinsically disordered proteins with potential adaptive value with regard to tolerance to freeze-induced cell desiccation. They are defined by the presence of one or more Lysine-rich K segments that can be combined with other conserved domains including Y segments located at the N terminus. Bulk segregant analysis (BSA) of alfalfa (*Medicago sativa* spp. *sativa*) populations recurrently selected for superior freezing tolerance uncovered a Y2K4 dehydrin restriction fragment length polymorphism (RFLP) with a demonstrated impact on freezing tolerance [Rémus-Borel et al. (2010) TAG 120:1163]. Investigation of intragenic polymorphism identified a subgroup of cold-inducible Y2K4 size variants that were more intensely amplified in genotypes with the RFLP and in response to recurrent selection. A detailed analysis of the association with the freezing tolerance phenotype led to the identification of a paralog with a characteristic short intron within that group of size variants, that responded positively to selection [Castonguay et al. (2012) TAG 124:809]. Sequence-characterized amplified region (SCAR) primers targeting the intronic region confirmed a stronger amplification of the short-intron variant in genotypes with the RFLP likely as a result of copy number variation. The recent discovery of a cold-inducible K3 dehydrin variant that co-segregates with the RFLP and the Y2K4 short-intron variant suggests the presence of a genome domain where genes that share functional and sequence similarity are clustered and co-regulated. Our results illustrate that enrichment of favourable alleles by recurrent selection facilitates the identification of functional variants in crops with limited genomic resources.



## Isolation and identification of cold resistance genes from *Medicago falcata*

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*Medicago falcata* is closely related to *M. sativa*, but exhibits greater tolerance to cold. myo-Inositol phosphate synthase (MIPS) catalyzes synthesis of myo-inositol, while galactinol synthase (GolS), using myo-inositol as substrate, catalyzes formation of galactinol that is synthesized to raffinose family oligosaccharides (RFOs). myo-Inositol, galactinol and RFOs play an important role in abiotic stress tolerance. During cold acclimation higher levels of myo-inositol, galactinol, raffinose, and stachyose accumulated in *M. falcata* than in *M. sativa*. MfMIPS1 and MfGolS1 were therefore isolated from *M. falcata* and characterized. In vegetative tissues under room temperature condition, weak MfMIPS1 transcripts were detected, and MfGolS1 could not be detected using RNA blot hybridization; however, they were highly induced in leaves after cold treatment for 24 h. The time course examination showed that transcripts of MfMIPS1 and MfGolS1 were induced earlier and maintained higher in leaves of *M. falcata* than *M. sativa* in response to cold at 5 °C. MfMIPS1 was also induced by dehydration and salt stress while MfGolS1 was weakly induced by salt stress, but not responsive to dehydration. Pharmacological experiments revealed that MfMIPS1 was induced by H<sub>2</sub>O<sub>2</sub> and nitric oxide (NO), but not abscisic acid (ABA); MfGolS1 was induced by sucrose and myo-inositol, but not H<sub>2</sub>O<sub>2</sub>, NO, and ABA. H<sub>2</sub>O<sub>2</sub> and NO were demonstrated to be involved in cold- and dehydration-induced MfMIPS1 expression in a combined action. Transgenic plant analysis indicated the elevated levels of myo-inositol and galactinol were associated with the enhanced resistance to cold, drought and salt stresses in the transgenic tobacco plants overexpressing MfMIPS1; and the elevated galactinol was associated with the enhanced resistance to abiotic stress in the MfGolS1 overexpressing plants. The other cold resistance genes isolated from *M. falcata* will be also introduced.

## Genes Associated with Aluminum Tolerance in Alfalfa (*Medicago sativa*): Variation in Sequence and Expression Levels

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Aluminum (Al) toxicity is an abiotic factor limiting crop productivity in acid soils. Acid soils account for 40% of the world's arable land. Alfalfa (*Medicago sativa* L.) is the most widely grown forage legume and an important component of sustainable agricultural systems. Alfalfa productivity is limited by Al toxicity in acid soils. Biomass yield and persistence are compromised due to the inhibition of root growth in acid and Al-toxic soils. Quantitative trait loci (QTL) for Al tolerance were identified in a mapping population resulting from a cross between Altet-4 (Al tolerant) and NECS-141 (Al sensitive) using molecular markers. The objectives of this study were to identify candidate genes associated with Al tolerance in tetraploid alfalfa and to evaluate variation in the gene sequences (SNPs) and their expression patterns in Al sensitive vs. Al tolerant alfalfa germplasm. High resolution melting (HRM) analysis was used to genotype 119 SNPs developed from genes differentially expressed between *Medicago truncatula* accessions grown with and without Al. SNP markers targeting candidate genes were integrated into alfalfa linkage maps and their association with Al tolerance QTL was evaluated. Results from our current progress to evaluate differential gene expression of candidate genes in alfalfa with acid and Al tolerance will be presented. Understanding mechanisms of Al tolerance and the underlying genetic mechanisms can facilitate the implementation of molecular breeding strategies and biotechnology approaches to develop alfalfa cultivars that are productive in acid and Al toxic soils.

# Genomic Selection in Perennials

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Breeding perennial forage, turf, and biofuel crops is a slow process, requiring multiple years to complete each cycle or generation of selection. This is exacerbated when the principal selection criterion is a complex trait of huge economic value, such as turf quality, forage yield, or biomass yield, that must be measured under realistic field conditions that include competition, multiple sites, and multiple years (ages of stand). Adding juvenility and vernalization requirements, which compound this problem for many species, results in cycle times that often exceed 5 years. Recent advances in DNA sequencing technologies have created opportunities to apply genomic selection technologies to a small number of economically important perennial crops. Genomic selection involves saturation of the genome with single-nucleotide polymorphic (SNP) markers to a level that generates linkage disequilibrium between most of the important quantitative trait loci (QTL) controlling the trait of interest and one or more SNP markers. Genomic selection offers two distinct advantages to perennial forage, turf, and biofuel crops: (1) it creates a ready mechanism to conduct meaningful selection among plants within families for traits that cannot be effectively measured on a single-plant basis (e.g. turf quality, forage yield, and biomass yield under competitive conditions) and (2) it offers an opportunity to drastically reduce the average time required to complete a cycle of selection. Genomic breeding values (GEBV) are computed as the sum of all SNP marker effects, capturing as much variation as possible for the relevant QTL. The genetic correlation between GEBV and phenotype, often termed the accuracy of GEBV estimation, is one of the critical keys to the success and efficiency of genomic selection. Early estimates suggest that accuracies of 0.3 to 0.5 could result in doubling of selection gains for perennial grasses and legumes.

## Towards genomic selection in perennial ryegrass

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Genomic selection (GS) attempts to make use of the availability of increasing amounts of genotypic information by enabling the prediction of breeding values for quantitative traits based on determining the effect of a large number of markers simultaneously. The aim is to enhance the efficiency of breeding programmes by increasing the speed of a breeding cycle, reducing the cost of phenotyping, and accelerating selection of candidates for production of synthetics. The perennial ryegrass breeding programme at IBERS differs from many others by its longevity and small size of its base population. In theory, this decreases the number of markers needed to achieve a certain level of prediction accuracy. I will discuss the opportunities for incorporating GS into this breeding programme, and describe preliminary information on linkage disequilibrium, and some cross-validation accuracies based on 3000 DArT markers, and highlight some of the challenges associated with GS. Ten-fold cross-validations within each of two breeding populations were surprisingly variable among sets, but also varied between traits. Variation spanned between 0 and 0.6, with the majority between 0.3 and 0.4. More consistent prediction accuracies were achieved with a larger training population of 288 genotypes derived from three varieties. The phenotypic data in this case was based on spaced plants, while the predictions in the breeding populations were projected from plot data of half-sib progeny.

# Genetic Characterization and Linkage Disequilibrium in five *Lolium perenne* Populations using Genic SNP Markers

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Predicted climate changes with higher temperatures and longer growth will make it possible to extend the cultivation of perennial ryegrass further north in Europe. However, climate change might lead to more unstable winter climates and winter survival of perennial ryegrass need to be improved in order to be successful in these regions. Using a population mixtures of 5 diverse populations, i.e. two European cultivars (Arsenal and Toronto), one Norwegian cultivar (Fagerlin), and two Norwegian breeding populations (FuRa0575-79 and FuRa9805), grown at several field locations covering a wide range of latitudes and climatic conditions in Norway, we aim to establish marker-trait associations by investigating shifts in allele frequencies of molecular markers. In order to establish the number and distribution of markers necessary for detecting allelic shifts we estimated linkage disequilibrium (LD) within and among the cultivars/populations. Forty genotypes from each of the 5 cultivars/populations were genotyped at 278 genic SNP loci selected across seven linkage groups. In general low LD was observed and LD decay was rapid although some differences were observed among cultivars/populations and linkage groups. As expected LD was correlated with the effective population size; lower LD was observed in Arsenal (based on a polycross between 40 clones selected from the cultivars Abersilo and Respect) compared with Toronto (based on a polycross between 10 clones from the population SLp 91-136). Analysis of population structure indicated two sub-populations within Arsenal and Fura9805, and three sub-populations within Toronto, Fura0575-79 and Fagerlin. Allele frequencies, genetic diversity, polymorphic information content were estimated within the cultivars/populations and will be presented. This is the first report of genome wide high throughput genotyping using genic *L. perenne* SNPs. Strategies for detecting allelic shifts in samples from field populations will be discussed.

## Structure-Function Analysis of Caffeic Acid O-Methyltransferase from Perennial Ryegrass (*Lolium perenne* L.)

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Lignin forms from the polymerization of phenylpropanoid-derived building-blocks (the monolignols); the modification through hydroxylation and O-methylation of the monolignols modulates the chemical and physical properties of the lignin polymer. The enzyme caffeic acid O-methyltransferase (COMT) is central to lignin biosynthesis, and is often targeted transgenically to alter plant lignin composition. Despite intensive investigation, the structural determinants of the regiospecificity and substrate selectivity of COMT remain poorly defined. Reported here are X-ray crystallographic structures of perennial ryegrass (*Lolium perenne* L.) COMT (LpOMT1), in open conformational-state, apo- and holo-enzyme forms; and most significantly, in a closed conformational-state complexed with the products S-adenosyl-L homocysteine and sinapaldehyde. The product-bound complex reveals for the first time the post-methyl-transfer organization of COMT's catalytic groups with reactant molecules, and the fully formed phenolic-ligand binding site. The core scaffold of the phenolic ligand forges a hydrogen-bonding network involving the 4-hydroxy group that anchors the aromatic ring and thereby permits only meta hydroxyl-groups to be positioned for transmethylation. While distal from the site of transmethylation, the propanoid-tail substituent governs the kinetic preference of ryegrass COMT for aldehydes over alcohols and acids due to a single hydrogen-bond donor for the C9 oxygenated moiety dictating the preference for an aldehyde.

# Development of an integrated transcript sequence database and a gene expression atlas for gene discovery and analysis in switchgrass (*Panicum virgatum* L.)

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Switchgrass (*P. virgatum* L.) is a perennial C<sub>4</sub> grass with potential to become a major bioenergy crop. To help realize this potential, a set of RNA-based resources were developed. Expressed sequence tags (ESTs) were generated from two tetraploid switchgrass genotypes, Alamo AP13 and Summer VS16. Over 11.5 million high-quality ESTs were generated with 454 sequencing technology and an additional 169,079 Sanger sequences were obtained from the 5' and 3' ends of 93,312 clones from normalized, full-length-enriched cDNA libraries. AP13 and VS16 ESTs were assembled into 77,854 and 30,524 unique transcripts (unitranscripts), respectively, using the Newbler and PAVE programs. Published Sanger-ESTs (544,225) from Alamo, Kanlow, and 15 other cultivars were integrated with the AP13 and VS16 assemblies to create a universal switchgrass gene index (PviUT1.2) with 128,058 unitranscripts, which were annotated for function. An Affymetrix cDNA microarray (Pvi\_cDNAa520831) containing 122,973 probe sets was designed from PviUT1.2 sequences, and used to develop a Gene Expression Atlas for switchgrass (PviGEA). The PviGEA contains quantitative transcript data for all major organ systems of switchgrass throughout development. We developed a web server that enables flexible, multifaceted analyses of PviGEA transcript data. The PviGEA was used to identify a full-complement of genes involved in monolignol biosynthesis.

## Transcriptional factor LcDREB2 cooperates with LcSAMDC2 to contribute to salt tolerance in *Leymus chinensis*

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S-Adenosyl-methionine decarboxylase (SAMDC) is a key enzyme in PA biosynthesis and catalyzes the conversion of S-adenosyl-methionine (SAM) to decarboxylated S-adenosyl-methionine (dcSAM). Dehydration responsive element-binding proteins (DREBs) exert protective effects under stress by activating the expression of downstream genes. SAMDC and DREBs have been extensively studied in improving plant resistance to abiotic stresses, but the mechanism for transcriptional regulation of SAMDC remains unclear. In this study, the LcSAMDC2 gene and its promoter were isolated from *Leymus chinensis*, a perennial rhizome grass distributed in the eastern Eurasian steppe (including the northern Plain) and the Inner Mongolian Plateau of China, with important roles in environmental protection and livestock development. Two DRE cis-elements were identified from the promoter of LcSAMDC2 and shown to bind LcDREB2. Subcellular localization and a yeast one-hybrid assay revealed that LcDREB2 is a transcription factor. An EMSA (Electrophoretic Mobility Shift Assay) showed that LcDREB2 can bind to the LcSAMDC2 promoter probe containing a DRE element. Over-expression of LcDREB2 in *L. chinensis* calluses increased expression of LcSAMDC2. Co-expression of LcDREB2 and the promoter of LcSAMDC2 fused with GUS in tobacco activated GUS activity. These results indicate that LcSAMDC2 is one of the downstream genes of LcDREB2. In addition, transgenic expression of LcDREB2 and LcSAMDC2 in *Arabidopsis* can improve the salt stress tolerance of transgenic lines. Our results indicate that LcDREB2 cooperates with LcSAMDC2 to contribute to resistance to abiotic stress.

# Commercialization of GE Traits in Forages

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Numerous novel traits have been identified and introduced into cultivated crops through conventional breeding, including the use of interspecific crosses and mutagenesis. Genetic engineering has offered additional opportunities to plant breeders to introduce novel genetic variation for traits and characteristics that was previously not available using only those more conventional plant breeding tools. As of January 1, 2012 over 90 genetically engineered (GE) trait/crop combinations had been fully deregulated and were available for commercial sale in the United States (Smith, 2012). The vast majority of these are traits associated with four commercial crops: corn, soybeans, canola and cotton. The commercialization of GE traits in perennial forage crops has introduced some incremental complexity when compared with the annual row crops that have been commercialized with GE traits to date. Perenniality *per se* complicates the extensive testing required for USDA, FDA and/or EPA deregulation of GE traits. In addition, the polyploid nature of many of the widely used forage species also complicates breeding schemes and variety development programs when high purity levels of the GE trait are required. For species requiring insect or wind mediated open-pollinated seed production, research on pollen flow has been critical to effectively design stewardship programs that facilitate the seed production and commercial coexistence of GE-traited varieties, conventional varieties and organic seed for both domestic and export markets. Alfalfa is the first perennial forage crop commercialized with a GE trait. GE alfalfa provides examples of the challenges outlined above, and also provides some possible solutions to these challenges. I will present the experience that Forage Genetics has gained with the introduction of Roundup Ready Alfalfa as a case study for discussing challenges and opportunities in commercialization of GE traits in perennial forages.

## Advances in genetic modification of switchgrass

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Genetic improvement of switchgrass (*Panicum virgatum* L.) through biotechnological approaches is expected to play a crucial role in modifying the quality or quantity of biomass suitable for biofuel production. Development of genetic tools is essential for effective improvement of existing switchgrass cultivars. Switchgrass, like many other grasses, is generally considered difficult to genetically manipulate at the cellular level. The establishment of a well-defined, rapid and highly efficient genetic transformation system is an important prerequisite for genetic engineering of this species. We have developed a highly efficient transformation system for the widely used switchgrass cultivar Alamo by identifying genotypes that are highly responsive to tissue culture and optimizing transformation parameters. The transformation efficiency of this system (number of independent transgenic plants/number of calli used for infection) exceeds 90%. Moreover, the system is consistent, highly reproducible and has facilitated the reduction of recalcitrance and increased biomass yield in switchgrass. For example, the genetic modification of lignin biosynthesis genes resulted in transgenic plants with improved sugar release and increased ethanol production. The overexpression of microRNA156b resulted in significant improvements in biomass yield. The development of an enhanced transformation system resulted in significant advances in the genetic modification of switchgrass, ultimately expediting the improvement of switchgrass for cellulosic biofuel production.

## Application of proteomics and transgenesis for the improvement of forage plants

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Forage productivity is affected by various environmental stresses such as salinity, heavy metal, drought, and extreme temperature. Therefore, understanding of stress biology is essential. Proteomics is an advantageous area of study in dissecting stress response mechanisms to boost our understanding of systems-level cellular behavior, especially in forage plants where we lack genome sequence information. Plant genetic engineering is also benefited from this knowledge by the identification of new gene products that may confer stress tolerance. We studied the rice proteome in response to heat and heavy metal stresses leading to the discovery of some promising candidates for stress tolerance such as a mitochondrial HSP and GST omega. In addition, we constructed a reference map for the leaf proteome of *Miscanthus sinensis*. This information will provide the basis for future studies to elucidate stress response mechanisms. Among the potential genes found by proteomics, we overexpressed *Oshsp24* in *Arabidopsis* and *Oshsp26* in tall fescue. Transgenic tall fescue plants showed greater photosynthetic capability and overall stress tolerance under heat stress compared to wild-type plants. Additionally, we generated transgenic tall fescue plants overexpressing chaperone (2-Cys Prx) and signal pathway-related gene (AtNDPK2), under the control stress-inducible promoter. The transgenic plants maintained photosynthesis and showed enhanced tolerance to several abiotic stresses such as methyl viologen, hydrogen peroxide, NaCl or heavy metals. The potential contribution of proteomics towards biotechnology and the value of genetic transformation for improving forage crops against common environmental stresses will be discussed. (This work was supported by a grant from the Next-Generation BioGreen 21 Program (No. PJ008139), RDA, Korea).

# Poster Abstracts

## Assessing the genetic diversity and reproductive strategy of *Danthonia spicata* through SSR marker analysis

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*Danthonia spicata* is a native cool-season grass often found in managed turf environments throughout the east coast of the United States. Commonly known as poverty oatgrass *D. spicata* is often associated with low pH high iron soils and has shown excellent tolerance of shade, drought, and low soil fertility. Currently very little is known about the level of genetic diversity or the primary reproductive strategy (outcrossing, self-fertilization or apomixis) in *D. spicata*. High throughput 454 sequencing of random sheared *D. spicata* genomic DNA was used to develop a Simple Sequence Repeat resource of approximately 450 SSR containing fragments. Primers were designed to 96 dinucleotide repeat containing fragments and screened on a progeny population developed from a single turf type *D. spicata* plant. Approximately 60 of the 96 primer pairs tested produced fragments in the expected size range and of these 60 none have shown evidence of segregation. Ten primer pairs that produced repeatable uniform amplification were chosen for analysis of genetic diversity levels of *D. spicata* populations from the Washington D.C. area. Preliminary results indicate limited genetic variation exists within and between these selected populations. The implications for future breeding efforts will be discussed.

## Biochemical response of some Iranian native grasses under drought stress

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Native cool and warm-season grasses are important for the enhancement of arid and semiarid rangeland in Iran. *Agropyron* (Dewey and Asay, 1975), *Festuca* (Rechinger, 1970) and *Cynodon* (Hralan and Rawal, 1970) grasses are highly distributed in Iran. Previous studies have established that changes in protein synthesis or degradation may influence drought tolerance (Jiang and Huang, 2002). Drought tolerance is also accompanied by antioxidant metabolism in various plant species (DaCosta and Huang, 2007). Our objective was to compare biochemical responses of Iranian native grasses under drought stress in a greenhouse study. Seeds from 3 populations of native cool-season and one clone of a warm-season grass species were collected from rangeland habitats of Iran. Seeds of *Agropyron cristatum*, *Festuca ovina*, *Festuca arundinacea* and a single *Cynodon dactylon* sprig with four nodes were planted in pots (25 cm in diameter and 30 cm deep). An increase in total soluble carbohydrate and sucrose content in the non-irrigated treatment was observed in *Festuca arundinacea* (Fig. 1). Martinez et al. (2003) indicated that under drought stress *Lolium perenne* releases sucrose for osmotic adjustment. Our result shows that carbohydrate content in *Cynodon* under 50% FC was noticeably higher than under 75% FC (Fig. 1.A). All non-irrigated treatments of *Cynodon dactylon* died and was not included in the graph. Hays et al. (1991) found an increase in soluble carbohydrates in response to drought stress in *Cynodon* to be associated with morphological root changes (drought avoidance) but in our study no relationship was observed between *Cynodon dactylon* turf quality and carbohydrate content under drought stress. Based on responses to drought, and the magnitude of total carbohydrate, protein and antioxidant activity, *Festuca arundinacea* was superior to other species in drought tolerance.



## Molecular and physiological analysis of two salt-tolerant alfalfa (*Medicago sativa*) lines selected for us on semi-arid rangelands

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Soil salinity is a growing global concern and in semi-arid regions saline concentrations can severely limit or prevent alfalfa production. Three cycles of recurrent selection for survival at EC=18 dS m<sup>-1</sup> in a greenhouse screening was used to develop two sativa-type and two falcata-type populations of alfalfa with increased salt tolerance. The selected alfalfa populations were field tested under saline and non-saline conditions. Several selected lines had minimal reduction in forage yield under non-saline conditions, and greater survival and yield than the parents under saline conditions. One sativa-type line, CS15-2, and one falcata-type line BC11-1 were chosen for physiological and molecular analysis. Measurements of several physiological parameters were conducted on greenhouse grown plants, including stem length elongation, leaf number, relative water content, and total electrolyte content. Plants from both CS15-2 and BC11-1 had greater stem elongation and maintained high number of leaves under salt treatment compared to their parents. Measures of the chlorophyll content index showed that both selected lines accumulated more chlorophyll than the parents under salt stress, and improved ability to maintain water status (relative water content). CS15-2 showed little accumulation of electrolyte while the parents showed a significant increase in electrolyte after salt treatment. However, BC11-1 showed the same increase of electrolyte accumulation as its parents. The results imply that CS15-2 and BC11-1 may have different mechanisms of salt tolerance. Additional physiological analyses are underway to better define the mechanism(s) of salt tolerance in these lines. Furthermore, microarray analysis using the *Medicago* GeneChip was used to measure gene expression of CS15-2 and BC11-1 alfalfas, and their parents under control and salt conditions.

## Molecular characterization of two bermudagrass populations for winter survival using genomic SSR markers from common bermudagrass (*Cynodon dactylon* var. *dactylon*)

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Bermudagrass, *Cynodon* spp, is economically important as a major turf and forage species in the southern United States. However, in transition zones and higher altitude areas, winter survivability is a major concern. Freeze tolerance in bermudagrass is a heritable trait. Limited SSR markers are currently available for bermudagrass research. The objective of this study was to analyze two bermudagrass populations using genomic SSR markers. Within each population they will be further analyzed on the basis of winter survivability by selecting plants that survived the winter and comparing them with the original plants within the given population. Genomic DNA samples were extracted from the bermudagrass genotypes 'RV' and 'YK'. Five libraries were constructed for SSRs enriched for CA-, GA-, ATG-, AAC- and CAG-sequences. A total of 44 non-redundant highly amplified polymorphic markers from all five libraries were used in this study. Results indicate that RV winter survival selection population produced significant different banding patterns than the original population in 86.4% of the SSR markers used. YK data showed a significant difference between winter survival and original population in 36.4% of the SSR markers used. It is interesting to note that RV was significantly different from YK in 34.1% of the markers used. This data suggests the two germplasms used in this study, should be used in breeding new cold tolerant bermudagrass cultivars.

## Proteomic analysis of *Miscanthus sinensis* leaves subjected to heat stress

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*Miscanthus sinensis* is a fertile perennial rhizomatous C<sub>4</sub> grass that has been traditionally used as forage and fibers. The recent focus on the use of renewable biomass as a possible alternative to fossil fuels has established a role of this carbon neutral plant as a bioenergy feedstock. Naturally, *M. sinensis* is exposed to high temperature during its main growing season. Therefore, understanding the heat stress response of *M. sinensis* would be useful for the development of heat-tolerant crop plants in the future. Until now, the plant is poorly represented in genome sequence databases. Proteomics has several advantages over other mRNA-based approaches in non-model organisms to study cellular processes at the molecular level. We exposed the seedlings to heat stress of 42°C in a growth chamber for 24 h and 48 h. A group of seedlings treated for 48 h were further allowed to recover for next 48 h. Total proteins were extracted from leaves and analyzed by two-dimensional gel electrophoresis. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) or MALDI-TOF/TOF MS allowed to identify 56 differentially expressed protein spots in heat-treated leaves. These proteins were involved in a variety of cellular processes including photosynthesis, energy metabolism, heat shock responses, signal transduction, transcription and protein synthesis. Interestingly, unlike previous studies, C<sub>4</sub>-specific pyruvate orthophosphate dikinase, Rubisco large subunit and its associated proteins were up-regulated during heat stress and tend to restore upon recovery. Identification of these proteins revealed some important clues regarding the way *M. sinensis* copes with high temperature conditions.

## Expression and functional characterisation of a white clover isoflavone synthase in tobacco

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Productivity of *Trifolium repens*, a valuable component of temperate pastures, is reduced by insect pests. It has been suggested that phenylpropanoid derived isoflavonoids such as formononetin can protect white clover from insect damage. Isoflavonoids are also involved in the establishment of root nodulation. Formononetin and its breakdown product, equol, are phytoestrogens. The aim of this study was to isolate and functionally characterise an isoflavone synthase (IFS2\_12) from *T. repens* by expressing it in *Nicotiana tabacum* (tobacco). To induce anthocyanin production and increase isoflavonoid precursors in tobacco we expressed the tomato R2R3 MYB transcription factor ANT1 in tobacco. We heterologously expressed IFS2\_12 in tobacco both transiently and stably, and analysed isoflavonoids in leaf extracts by liquid chromatography coupled to mass spectrometry. As a positive control we also expressed a double construct of soybean IFS and alfalfa chalcone isomerase (IFS/CHI) which had been previously shown to induce isoflavonoid production in tobacco. Stable transformants expressing IFS2\_12, IFS/CHI and ANT1 were crossed and resulting plants analysed for isoflavonoid production. Leaves of tobacco plants expressing ANT1 had a range of tissue colour phenotypes from green through bronze to purple. Both transient and stable expression of the IFS2\_12 or IFS/CHI constructs resulted in the production of the isoflavonoid genistein and its conjugates. Highest levels (up to 19.2 mg g<sup>-1</sup> DW) accumulated in a progeny of a purple ANT1 crossed to a IFS/CHI transformant, while the second highest was found in a plant derived from a selfed IFS2\_12 transformant. We conclude that the gene IFS2\_12 isolated from *T. repens* encodes an isoflavone synthase. This study paves the way for engineering white clover plants with isoflavone levels sufficient for insect protection.

# Development and Application of Duplex PCR for the Identification of Selfed Progeny in Switchgrass

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Switchgrass is a herbaceous model plant for the cellulosic biofuel feedstock development in the USA and Europe. Accurate identification of selfed progeny is important to produce switchgrass inbreds, which can be used in the production of heterotic hybrids. Development of a technically reliable and easily used marker system is required to quantify and characterize breeding origin of progeny plants of targeted switchgrass parents. Here a genome-wide screening of 915 mapped microsatellite markers was conducted, and 842 (92%) produced clear and scorable bands on a pooled DNA samples including eight major switchgrass varieties. A total of 166 primer pairs were selected on the basis of their evenly distribution in switchgrass genome and PCR amplification quality on 16 tetraploid genotypes. Mean polymorphic information content value for these 166 selected loci was 0.810 ranging from 0.116 to 0.959. Among them, a core set of 48 loci, which evenly distributed on 17 linkage groups and had mean null allele frequency of 0.059 with a range from -0.196 to 0.281, suggesting low mutation rates, was further refined and optimized to develop 24 sets of duplex markers. Most of (up to 87.5%) non-allelic bands within each duplex were separated by more than 10-bp. Assuming one known parent, 10 randomly selected markers provided combined non-exclusion probability of less than 0.0001 (i.e., accuracy being > 99.99%) in the identification of selfed progeny. Using the established duplex PCR protocol, selfing ratio was 0 for an open-pollinated 'Kanlow' genotype, 11.3% for ten selected and bagged parents, and 77.3% for a breeding line grown in a growth chamber. In conclusion, the duplex PCR of 48 loci provides ample choices for unlinked loci on switchgrass whole genome, and represents a powerful and reliable method for the identification of selfed progeny in switchgrass.

# Genetic Engineering of Sugarcane for Increased Fermentable Sugar Yield from Lignocellulosic Biomass

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Sugarcane is one of the most efficient photosynthesizer in the plant kingdom, able to convert as much as 2% of incident solar energy into biomass. In the U.S. sugarcane is mainly grown for the production of sugar with Florida being the largest producer of sugarcane, followed by Louisiana, Hawaii, and Texas. A large amount of lignocellulosic biomass such as leaf litter residues and bagasse are generated during the sugarcane harvest or after the sugar refining process, respectively. Therefore, lignocellulosic biomass from leaf and processing residues will likely become a valuable feedstock for biofuel production. However, higher temperatures and/or acid concentrations result in dehydration of xylose to furfural, and glucose to hydroxymethyl furfural, which act as inhibitors of the fermentation process. New pretreatment protocols are being developed that require the application of xylanases and other enzymes for maximal yields of xylose. Our objectives target the improvement of fermentable sugar yields from hemicellulosic sugarcane residues and enhancing the biosafety of the transgenic plants. We evaluated two transgenic approaches: lignin modification by RNAi suppression of the lignin biosynthetic gene COMT and in planta production of a hyperthermostable xylanase. More than 200 transgenic sugarcane plants were generated and lines with suppression or expression of the target genes were selected. RNAi suppression of COMT resulted in reduced lignin content and altered lignin composition. In planta produced xylanase Xyl10B converted the majority of sugarcane xylan to fermentable xylobiose. Performance and conversion efficiency of transgenic plants grown in replicated field plots under USDA-Aphis notification 11-040-120 will also be presented.

# Genetic Improvement of Elephantgrass (*Pennisetum purpureum* Schum.) through Breeding and Biotechnology

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Elephantgrass (*Pennisetum purpureum* Schum.) is the most productive perennial biomass grass for the Southern US and a prime candidate for production of lignocellulosic biofuel. Biomass yield and low input characteristics like nutrient uptake efficiency and abiotic and biotic stress tolerance are the main targets for genetic improvement of elephantgrass. Our research involves molecular marker analysis for the identification of superior parents and confirmation of crosses, intra and interspecific hybridization and selection of superior hybrids, chromosome doubling to restore fertility of triploid interspecific hybrids and genetic transformation to introduce transgenes for suppression of flowering, improved stress tolerance and quality. For the development of the first genetic transformation protocols for elephantgrass and sterile, interspecific hybrids of elephantgrass and pearl millet tissue culture, gene transfer and selection protocols for various explants including immature leaves, inflorescences and seeds were optimized. For chromosome doubling an in vitro protocol which improved the production of non-chimeric plants was developed. Interspecific hybridization between elephantgrass and pearl millet (*Pennisetum glaucum*) supports improvement of important traits (e.g. drought tolerance, biomass quality and production). In contrast to elephantgrass, which produces large amounts of wind dispersed seeds, interspecific hybrids are male and female sterile due to triploidy ( $2n = 3x = 21$ ) and do not produce seeds which contributes to biosafety. Chromosome doubling of the triploid hybrids restored seed production and fertility, and allowed backcrosses with elephantgrass to generate pentaploid, interspecific hybrids for further improvement of biomass production and persistence. Intra and interspecific elephantgrass hybrids with high agronomic performance were selected under field conditions. Chromosome doubling was obtained for all interspecific hybrids with superior biomass production and for the first time the generation of transgenic elephantgrass is reported.

## Genetic modification of lignin in switchgrass reduces recalcitrance and improves ethanol production and forage digestibility

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Switchgrass is a leading dedicated bioenergy feedstock in the United States because it is a native, high yielding, perennial prairie grass with broad cultivation range and low agronomic input requirements. Biomass conversion research has developed processes for production of ethanol and other biofuels but they remain costly primarily due to the intrinsic recalcitrance of biomass. We show here that switchgrass genetic modification can produce phenotypically normal plants that have reduced recalcitrance to fermentative bio-processing. Downregulation of the switchgrass cinnamyl alcohol dehydrogenase (CAD) gene or caffeic acid O-methyltransferase (COMT) gene decreases lignin content modestly, reduces the syringyl to guaiacyl lignin monomer ratio, and improves sugar release. More importantly, the downregulated COMT lines showed up to 38% increase in the ethanol yield by using conventional biomass fermentation processes. They require 300-400% lower cellulase dosages for equivalent product yields using simultaneous saccharification and fermentation (SSF) with yeast. In addition to increased ethanol production, the transgenic switchgrass also showed increased forage quality, which is very beneficial for farmers since switchgrass can serve as a dual purpose (bioenergy/forage) crop. Thus, the transgenic approach has the potential to improve the economic viability of various bio-based fermentation-derived fuels by greatly improving the energy, cost, and land-use efficiency of their production. The innovative transgenic switchgrass illustrates the feasibility of developing energy crops specifically designed for industrial processing to biofuel.

# Overexpression of miR156 in switchgrass leads to improved biomass production

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Switchgrass (*Panicum virgatum* L.) has been developed into a dedicated herbaceous bioenergy crop. Biomass yield is a major target trait for genetic improvement of switchgrass. microRNAs have emerged as a prominent class of gene regulatory factors that has the potential to improve complex traits such as biomass yield. A miR156b precursor was overexpressed in switchgrass. The effects of miR156 overexpression on SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL) genes were revealed by microarray and quantitative RT-PCR analyses. Morphological alterations, biomass yield, saccharification efficiency and forage digestibility of the transgenic plants were characterized. miR156 controls apical dominance and floral transition in switchgrass by suppressing its target SPL genes. Relatively low levels of miR156 overexpression were sufficient to increase biomass yield while producing phenotypically normal plants. Moderate levels of miR156 led to improved biomass but the plants were non-flowering. These two groups of plants produced 58-101% more biomass yield compared with the control. However, high miR156 levels resulted in severely stunted growth. The degree of morphological alterations of the transgenic switchgrass depends on miR156 level. The improvement in biomass yield was mainly because of the increase in tiller number. Targeted overexpression of miR156 also improved solubilized sugar yield and forage digestibility, and offered an effective approach for transgene containment.

## Standardization of Switchgrass Sample Collection for Cell Wall and Biomass Trait Analysis

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As a native, low-input crop with high biomass production, switchgrass (*Panicum virgatum*) has become a favorable feedstock for the production of cellulosic biofuels in the United States. Many efforts are being made to improve the production of cellulosic biofuels from switchgrass. Protocols regarding analysis of switchgrass biomass have been established; however, the developmental stage of the materials being analyzed has varied depending on researchers' discretion, and no standardized harvesting procedure has been defined. Developmental stages have a large impact on the results of biochemical analyses. We propose a standardized procedure for switchgrass sample collection for cell wall and biomass analyses by describing various developmental stages of switchgrass, defining the R1 stage as the stage at which tillers should be collected, and providing a detailed description of how and what material should be analyzed. Such a standardized procedure will help to maintain consistency in switchgrass evaluation methods, enable comparisons of data obtained from different approaches and studies, and facilitate efforts towards improving switchgrass as a bioenergy crop.

## Variation in Sequences and Expression Levels of Lignin Genes in Alfalfa Stems

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Alfalfa is the most important forage legume with potential value as a lignocellulosic crop. Lignin content and composition in alfalfa affect both forage quality and processing efficiency for conversion to biofuels. The objectives of this study were to identify genes and transcription factors involved in lignification and to evaluate variation in both sequence and expression levels of these genes in alfalfa genotypes contrasting for lignin content. A total of 20,439 single nucleotide polymorphism (SNP) markers were identified in lignin genes and transcription factors from stem transcriptome sequences of 27 alfalfa genotypes. High-resolution melting (HRM) analysis was used to validate over 95% of a subset of the in silico predicted SNPs located on multiple alfalfa chromosomes and their corresponding allelic dosages. Lignin content variation was identified in six alfalfa genotypes based on UV-microscopy and wet chemistry analysis. Lignin content was negatively correlated with forage quality, total sugar content and processing efficiency in the alfalfa samples evaluated. Quantitative RT-PCR was used to assess the levels of gene expression that were associated with differences in lignin content of the genotypes evaluated. Gene-derived SNP markers were integrated onto existing linkage maps in alfalfa and are being used to identify genomic regions relevant to lignin content in segregating populations. The identification of SNP markers in genes affecting lignin accumulation can be used to develop alfalfa cultivars with improved forage quality and processing efficiency by implementing molecular breeding approaches.

## Characterisation of a Phosphate Transporter Gene from White Clover (*Trifolium repens* L.)

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Acid soils, pH 5.5 or lower, are one of the most significant limitations to agricultural production worldwide, affecting approximately 30% of the total land area. The primary site of root growth inhibition by Aluminium (Al) toxicity is focussed at the root apex, which is also the region most actively involved in Al tolerance responses mediated by organic acid secretion. Root secretion of organic compounds represents a net loss of carbon to the organism, making an inducible, localised mechanism of Al tolerance mediated by root tip secretion the most effective means to confer Al tolerance. Transgenic modification offers the potential to enable targeted gene expression in desired tissue types under specific conditions for maximal effect with minimal fitness penalty. Plant gene promoters are the key to enable such a precision strategy. A phosphate transporter gene (TrPT1) from white clover has been identified and fully sequenced along with its 5' regulatory sequences to putatively direct root-specific gene expression inducible under low inorganic phosphate conditions, found under acidic Al-rich soils. An in depth in silico analysis of the TrPT1 promoter has been performed revealing a high level of conservation of the transcription factor binding sites identified in *Arabidopsis thaliana* and *Medicago truncatula* PT1 and PT2 promoter sequences. From a collection of characterised transcription factor binding site motifs the P1BS element, which regulates response to P deprivation, was identified in all of the PT promoter regions along with other sites associated with root and nodule expression and auxin induction. The detailed characterisation of the DNA sequence of the TrPT1 promoter demonstrates its suitability and specificity as an inducible and root tip cell-specific promoter for deployment in transgenic approaches for engineering Al tolerance in forage legumes such as white clover and alfalfa.

# Comparative Genetics and Genomics of White Clover (*Trifolium repens* L.) and Subterranean Clover (*Trifolium subterraneum* L.)

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The availability of the complete genome sequence of the barrel medic (*Medicago truncatula* Gaertn.) facilitates comparative genomic studies between this and other legume species. In previous studies, a comprehensive map has been developed for white clover (*Trifolium repens* L.), and the first comparative analysis between maps of subterranean clover (*T. subterraneum*) and red clover (*T. pratense*) maps, and the genome of *M. truncatula*, has been performed. In this study, the approach has been extended to comparisons between the maps of white clover and subterranean clover, which is an obvious target for comparative genomic studies due to agricultural importance, phylogenetic position and relative low genomic complexity. Detailed comparison of subterranean and white clovers with *M. truncatula* has identified inter- and intra-chromosomal rearrangements and demonstrated conserved synteny between chromosomes. Results suggest extensive genome conservation within the Trifolieae tribe across a substantial period of evolutionary divergence. The data supports the hypothesis that genomes of closely related species share regions of copy number variation. The outcomes of this study will expedite progress in clover genomics, assist genetic marker development, and highlight areas of interest for future evolutionary and functional studies.

# Comparative Genomic Analysis of Five Diploid Grasses from the *Festuca-Lolium* Species Complex

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Species belonging to the *Festuca-Lolium* complex comprise many agriculturally important cool-season forage grasses including perennial ryegrass (*L. perenne* L.), Italian ryegrass (*L. multiflorum* Lam.), meadow fescue (*F. pratensis* Huds. syn. *L. pratense* Huds. [Darbysh.]) and tall fescue (*F. arundinacea* Schreb. syn. *L. arundinaceum* Schreb. [Darbysh.]). A fully sequenced and annotated genome from each species would be ultimately desirable to permit major advances in forage grass genetics and breeding, through the provision of a complete catalogue of gene and regulatory regions of a species, as well as tools for evaluation of genome-wide variation. However, the genomes of important *Festuca-Lolium* species are both large (haploid genome sizes = 2–4 Gb) and complex (as tall fescue is an allohexaploid) when compared to model grasses such as rice (*Oryza sativa* L. – 389 Mb) and *Brachypodium distachyon* L. (270 Mb), for which complete genome sequences are available. Lower coverage survey-sequencing of multiple species offers an alternative method for obtaining gene catalogues and also enables inter-specific genomic comparisons. Low-coverage whole genome sequence was obtained from five diploid species of the *Festuca-Lolium* complex: *L. perenne*, *L. multiflorum*, *F. pratensis*, *F. altissima* and *F. ovina*. Sampling was designed to include agriculturally important forage grasses, along with representatives of each major sub-genus within the species complex. Following de novo assembly, sampling of c. 80% of the gene-space was estimated, based on a comparison with predicted genes from *B. distachyon*. The complete chloroplast genome was also assembled for each species, enabling phylogenomic analysis of included taxa. This work has generated sequences for many functionally significant protein-coding genes and will enable an insight into the pan-genome of the *Festuca-Lolium* species complex. The generated sequence will also provide a framework for assembly of the hexaploid tall fescue genome in the future.



## From Models to Crops: Use of Resources in *M. truncatula* for Crop Improvement in Alfalfa

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Increased worldwide demands for food and fuel are driving forces to develop crops and cropping systems that are more productive with reduced inputs. Alfalfa is one of the most important cultivated forage legumes worldwide and is a close relative of the model legume *Medicago truncatula*. Integrating information from existing and emerging databases with translational genomics approaches provides opportunities to leverage knowledge from *M. truncatula* and other legumes, to develop crop improvement strategies in alfalfa. Genetic variation for drought and aluminum tolerance, phosphorus-use-efficiency and lignin content was identified in diverse *M. truncatula* inbred lines representing the genetic diversity in the USDA collection. Integration of alfalfa transcriptome sequences with the *M. truncatula* genome sequence and gene annotations and QTL mapping studies facilitates the identification of candidate genes located in genomic regions relevant to the target traits in alfalfa including abiotic stress and forage composition. Ongoing efforts include integration of multiple phenotyping strategies, metabolomics profiling, transcriptome sequences, and bioinformatics approaches to facilitate the development and implementation of molecular breeding methodologies that increase the efficiency of cultivar development in alfalfa.

## Self-incompatibility in ryegrass: Homing in on the genes of the S-locus complex

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Self-incompatibility (SI) is a genetic mechanism that prevents self-pollination by using a system of recognition between the pollen and the stigma. The genetic control of this mechanism in grasses is hardly understood. In perennial ryegrass (*Lolium perenne* L.), SI is controlled by at least two loci, S and Z (Lundqvist, 1954; Hayman, 1956) which have not been characterized in any grass species yet. The S-locus in perennial ryegrass has been mapped on linkage group 1 (Thorogood et al., 2002) but, to date, mapping approaches for gene isolation and cloning have been inconclusive. Using mapping populations especially designed to map the S-locus, fine-mapping has been conducted by genotyping over 10,000 plants using high resolution melting curve analysis (HRM). So far, the two closest recombinant flanking markers of the S-locus have been mapped at 0.06cM and 0.01cM either side of the S-locus region that contains 6 genes in the rice and 9 genes in the Brachypodium homologous regions. Using these markers as well as several non-recombinant markers, contiguous *Lolium* BAC clones covering this region have been isolated, sequenced and assembled in order to build up the physical region of the S-locus. Moreover, RNA-seq from stigma and pollen tissue, as well as stigmas pollinated with incompatible and -compatible pollen, has been conducted to corroborate expressed candidate genes and to identify the causative polymorphism that is the basis of S locus specificity.

# Systems Biology Analysis of Gametophytic Self-Incompatibility in Perennial Ryegrass

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Perennial ryegrass, which is an important pasture grass crop in temperate climates, possesses a gametophytic self-incompatibility (SI) system controlled by two loci (S and Z). Although the SI system is believed to contribute to the maintenance of genetic diversity in populations, it provides major obstacles to targeted molecular breeding. A comprehensive approach using a systems biology paradigm has been initiated to enable the identification of both the S and Z genes. For the purpose of genetic mapping in perennial ryegrass, 17 (S) and 10 (Z) SI-linked DNA-based markers were developed using comparative genetic means. A large-scale mapping population composed of 567 individuals was established. The perennial ryegrass SI loci were fine-structure mapped by genotyping and phenotyping of this population. The S and Z loci were mapped on linkage groups (LGs) 1 and 2, respectively. A comparative approach with the SI-linked markers revealed gene-based microsyntenic relationships for both loci between perennial ryegrass and model grass plant species, such *Brachypodium distachyon* and rice. This microsyntenic information supported the fine-scale genetic and physical mapping processes. Additional DNA-based markers were developed based on microsyntenic information to dissect the probable intervals containing the two loci. For physical mapping, BAC clones representing the target regions were isolated from a perennial ryegrass genomic library and sequenced with a high-throughput second-generation sequencing system. BAC-derived sequences were aligned based on microsyntenic information, and gene-like sequences were identified. In addition, high-throughput deep transcript sequencing of target reproductive tissues has been performed to assist identification and ranking of candidate genes for further investigation. The generation of transgenic perennial ryegrass plants for targeted modification of the expression of candidate genes for S and Z has also been progressed for highly qualified candidate sequences.

## Form for Forage Phenomics

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The science and technology of genomics has accelerated rapidly over the last decade with advances in analysis of plant genomes now far exceeding our ability to undertake high-throughput, high accuracy plant phenotyping. Phenomics has opened the opportunity to overcome this phenotyping bottleneck through accurate, quantitative, high-resolution phenotyping based on digital analysis of plant morphology, development and function. These “whole of lifecycle”, quantitative measurements of plant (shoot and root) growth, development and performance enable accurate phenotyping for a range of applications such as functional analysis of genes; transgenic trait dissection (including root traits); phenotyping of core germplasm collections to underpin novel allele discovery; phenotyping of transgenic plant genotypes with novel traits (single traits and trait stacks); phenotypic screening transgenic plants for field evaluation; transgenic event optimisation and germplasm development. A total of seven species of interest (five grass and three legume species) are being phenotypically screened under field and containment, controlled environment conditions, encompassing a range of annual and perennial species, as well as different symbiotic interactions (fungal endophytes in forage grasses, and rhizobial bacteria in forage legumes). A forage phenomics platform is being established including a range of imaging technologies - from RGB to hyper-spectral and from in camera to airborne imaging - to undertake non-destructive measurements over time of: shoot mass, leaf number, shape, angle, and other morphometric data, and leaf colour and senescence using visible spectrum images; leaf water and carbohydrate content using near infrared images; leaf temperature using far infrared images; removal of water from soil in pots using near infrared wavelengths; plant health assessment by measuring leaf greenness using RGB imaging and monitoring the state of leaf photosynthesis using fluorescent imaging.

# Accelerated Genomics in Allotetraploid White Clover (*Trifolium repens* L.) Based on High-Throughput Sequencing

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White clover is an important forage legume in temperate grassland agriculture, which would benefit from a broader uptake of molecular breeding technologies to deliver the next generation of elite cultivars. However, as a complex outbreeding allotetraploid ( $2n = 4x = 32$ ) species, white clover presents significant challenges with respect to development of genomic resources. Significant efforts have led to the characterisation of the sequence diversity within and between the two constituent sub-genomes of this species. In addition, extensive comparative genomics with the model legume species *Medicago truncatula* and *Lotus japonicus* has delivered a rationalised linkage group nomenclature system, as well as translational genomics data for the prediction of gene order and content. Proof-of-concept work has been performed in SNP discovery, with amplicons from selected genes undergoing cloning, sequencing and validation to differentiate genuine allelic (homologous) variation from homoeologous and paralogous sequence variation. In order to validate a reduction-to-practice for a high-throughput SNP discovery pipeline, large-scale resequencing of genic amplicons has been performed. A total of 195 gene amplicons were selected based on two rounds of primer design, initially from genes of potential agronomic significance, and subsequently on the basis of predicted genetic map location based on comparative genomics. The parental plants from two biparental mapping populations were used as the template DNA source, and amplified products were pooled and sequenced using GS FLX Titanium technology. The sequence data was reference assembled using Mosaik software, and a collection of 9,610 putative SNP loci were identified. These SNP loci represent an important resource for the development of a highly-multiplexed SNP assay system to correlate genotypic diversity with phenotypic variation.

## Assembly and Analysis of de novo transcriptome in perennial ryegrass

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Perennial ryegrass (*Lolium perenne* L.) is an important grass species for both forage and amenity purposes. Denmark is the world's largest exporter of grass seed and therefore it plays an important role in the country's economy. It is envisaged that breeding efforts may be enhanced with the assistance of new breeding technologies such as genomic selection. A major step towards genomic selection will be the availability of a reference genome, and efforts are underway within our group to deliver this. An important step in de novo assembly will be defining the gene set, and the availability of transcriptome sequencing data will greatly aid gene prediction and validation, and the development of functional markers for improved ryegrass breeding. Therefore, the goal of this study is to develop a de novo assembly of the perennial ryegrass transcriptome from the same inbred genotype being used for de novo genome assembly. Furthermore, we also conducted de novo assembly with other heterozygous genotypes to enable SNP discovery for marker-assisted selection (MAS). In this study we have isolated RNA from leaf, stem, inflorescence, leaf sheath, root and meristem from the inbred and heterozygous genotypes to get a good representation at the transcriptome and study tissue specific expression.

# Computational Tools for Genomics-Assisted Forage Plant Breeding

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With the advent of next-generation genotyping platforms, the ability to rapidly and cost-effectively genotype plants in commercial breeding nurseries has become realistic and practical. This capacity not only paves the way for future integrated genomics-assisted breeding programs for forages, but already has direct application in current breeding programs. Maintenance of genetic diversity in breeding nurseries is fundamental for achievement of continual genetic gain in breeding lines. Loss of rare beneficial alleles can be common when intense phenotypic selection for several key traits is applied, which can present significant issues in future breeding cycles. In addition, the ability to perform population identification and seed purity analysis from genotype data for a quality-assured seed supply chain also provides benefits to breeders. However, rates of development in genotyping technology have not been matched by corresponding advances in computational tools to assist and enable their application. Selection while Conserving Diversity (SeConD), is a software package that assists breeders to select a sub-set of phenotypically elite individuals from a breeding nursery, whilst ensuring that all molecular marker alleles identified within the nursery are present in the selection. Statistical Analysis of Mixed Ploidy Populations (StAMPP) allows users to calculate Nei's genetic distance and pairwise *F<sub>st</sub>* values with confidence intervals and p-values from single nucleotide polymorphism genotypic datasets derived from samples of mixed ploidy levels. Previously unavailable to mixed ploidy genotype analysis, StAMPP allows breeders to develop cultivar catalogues, explore breeding history, examine population structure and perform population differentiation, to protect the genetics of proprietary cultivars and to provide confidence on seed purity to farmers. These two packages provide an effective link between genotype data and its application in commercial plant breeding.

## Construction of a DArT marker resource for better adapted forage crops to climate change

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Climate change stresses breeding for better adapted forage crops to increasing cold and warm temperature as well as to more and more severe summer water deficit and winter flooding. However, most of forage crops are out-breeding polyploids; furthermore, they are commonly used as complex mixtures of grasses and legumes which performance and resilience of annual productivity result from intricate interactions at both, within and between, genetic levels. In this context, a whole genome tool is required to survey genetic diversity and change at various scales of time and space to develop more effective breeding methods. A DArT marker resource is currently under construction in the 4 major forage crops: *Dactylis glomerata* ssp., *Festuca arundinacea* ssp., *Lolium* sp. and *Medicago sativa* ssp., with special care to insure a fair balance across sub-species, genomes and ecotypes. The aim is to deliver about 8 000 public polymorphic markers within each complex of species. Although DArT markers have the limitations of dominant markers, they allow whole and dense genome coverage suitable to reveal strong genetic disequilibrium between highly phenotypically differentiated populations, opening to introgression assisted marker breeding for complicated adaptive traits. DArT markers have been already applied in various fields of *Festuca* x *Lolium* breeding: cytogenetics, interspecific genome balance across cultivars and generations, freezing tolerance association as well as short-term genetic responses to water stress. Response to an artificial increase of temperature and summer water deficit is currently investigated between and within Temperate and Mediterranean cultivars of tall Fescue and Dactylis. Applied in mapping populations of *Medicago sativa*, DArT markers will help to rapidly saturate framework genetic maps from co-dominant SSR markers, and to more accurately localize QTLs. Finally, DArT markers let open access to gene level through DNA-sequencing and comparative genomics.

# Development of a Transcriptome Atlas for Perennial Ryegrass (*Lolium perenne* L.)

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The current generation of DNA sequencing platforms permits significant resources and data to be generated rapidly at relatively low cost. A single genotype of perennial ryegrass has been subjected to transcriptome analysis through deep sequencing of cDNA samples derived from multiple distinct tissue types. A total of 0.6 billion sequencing reads from the Illumina HiSeq2000 platform have been generated from 11 different vegetative samples, including leaf, pseudostem and root samples for both terrestrial and subterranean aspects of gene expression. DNA sequence assembly has been performed on the resulting data from each individual tissue sample, as well as an overall assembly using the SOAP de novo package version 1.05. The assembly generated between c. 23,000 and 29,000 scaffolds from each tissue type, and the combined assembly obtained 57,480 scaffolds, summing to in excess of 47 Mb, with an average length of 820 bp. The resulting contigs have been compared, using a BLAST analysis pipeline, to the gene complement from the related model grass species *Brachypodium distachyon* L. In excess of 85% of all predicted genes and splice variants of the fully sequenced model grass species have been identified in the assembled ryegrass data set. In addition, analysis of the data has enabled tissue specific patterns of expression to be identified, with over 23,000 genes attributed to a broad expression profile, along with smaller cohorts that display tissue-specific expression. The identification of specific transcription regulation patterns will be valuable for candidate gene identification for agronomically relevant traits.

## Improving Association Analysis of Stress Tolerance Traits by Model Testing and Selection in Perennial Ryegrass

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Population structure and/or relative kinship in the association mapping population can often result in many false positive association signals between gene and traits. Model testing and selection of the best model can increase accuracy of association mapping results. We conducted model testing and fitting for phenotypic traits related to drought tolerance and cold hardiness in 192 globally collected perennial ryegrass (*Lolium perenne* L.) accessions screened by 109 simple sequence repeat markers. Following the previously recommended procedures, simple linear model, Q (considering population structure), K (considering relative kinship) and Q + K models were tested with subpopulation membership percentage as fixed covariates and with kinship as a random effect. The best fit model was determined for each trait based on the value of Bayesian information criterion (BIC). The lower BIC indicated a better model fitting. We found that the Q model either had the lowest BIC value or had the second lowest BIC value for all the phenotypic traits in this mapping population. Approximately 83 % and 73 % of false positive correlations were eliminated after controlling population structure (Q model) for drought tolerance and cold hardiness, respectively; compared to the simple linear model. Our results demonstrate that model testing can largely eliminate noise signals in association mapping study of stress tolerance in perennial ryegrass.

## Rapid SSR Marker Development in Buffalograss

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Buffalograss (*Buchloe dactyloides*) is an important low input sustainable turfgrass species, native to North America. There are limited genomic resources available for studying buffalograss. Next generation sequencing provides a platform for increased access to sequence data at reduced costs and can be used to study species with little to no upfront sequence knowledge. The large amounts of sequence data generated from a single next generation sequencing experiment can be leveraged for expanding genomic resources and genetic marker development. In this study, 454 and Illumina sequencing was performed on Prestige and 378 buffalograss varieties. A total of 79.6 Mb and 9.5 Gb of transcriptome sequence data were generated from the 454 and Illumina sequencing respectively. A hybrid de novo assembly resulted in 560,720 Prestige and 356,547 378 contigs. After filtering the sequences based on length and sequence similarity criteria, 81,070 378 and 129,692 Prestige sequences remained. The software program RepeatMasker identified 3,748 simple sequence repeat (SSR) containing sequences from 378 and 7,611 from Prestige. Two and three bp repeats were the dominant SSR types. Reciprocal BLAST searches were performed and 1,586 homologous SSR-containing sequences were identified between the two varieties. In silico analysis was done to identify near identical sequences from Prestige and 378 that differed only by copy number of the simple sequence repeat. Flanking PCR primers were designed by Primer3 for each SSR and a subset was amplified on the parents from three experimental genetic linkage mapping populations. More than 92% of the SSR markers amplified a product in the expected size range. This approach allowed for rapid development of sequence specific SSR genetic markers and greatly increased the transcriptome sequence information available for future buffalograss studies.

## Whole Genome Sequencing of Perennial Ryegrass (*Lolium perenne* L.) Supports Exome Assembly for Gene and SNP Catalogue Development

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The current generation of DNA sequencing platforms enables significant resources and data to be generated rapidly at relatively low cost. Although determination of whole genome sequence for a complex plant genome is still a significant undertaking, generation and assembly of the exome (gene-encoding) component is possible with the use of more modest resources. A single plant genotype of perennial ryegrass has been sequenced to approximately 70 X coverage, with c. 2 billion sequencing reads of 100 bp paired-end sequence reads on the Illumina HiSeq2000 platform. The generated sequence data has been assembled using the SOAP de novo software package v 1.05. The sequence assembly has been empirically optimised through iterative assessment of performance based on a range of input kmer sizes, in terms of number of bases assembled, and the average length of assembled contigs and scaffolds. An optimal assembly has generated 1.9 million scaffolds covering c. 1.7 Gb, while all contigs and singletons cover c. 3.5 Gb. Comparison of the contigs and scaffolds to the coding sequences from *Brachypodium distachyon* L. has permitted identification of putative perennial ryegrass orthologues to c. 86% of all predicted genes and alternate transcripts from the model grass species. Development of this exome sequence library has enabled assembly and identification of a large collection of genic contigs, along with the corresponding regulatory elements. The collection is accessible to interrogation to characterise and document complete sets of gene families, along with provision of reference assemblies to identify sequence polymorphisms.

# Isolation and functional study of MsBAN gene in *Medicago sativa*

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Forages with high digestibility and abundant soluble proteins can cause pasture bloat, but condensed tannins can reduce the incidence and severity of bloat for ruminant livestock. The BANYULS (BAN) gene encodes a key enzyme in condensed tannin synthesis pathway. A cDNA of BAN gene was isolated from *Medicago sativa* using the RACE method. Conserved motifs were detected including a NADP binding site (VIGGTGFVASLLIKQLLEKGY) in the N-terminal region. A single-copy MsBAN gene was detected by Southern blotting *M. sativa* genomic DNA. Real-time PCR indicated that the expression level of BAN gene is highest in pods and least in flower. The expression of BAN gene was up-regulated by NaCl and PEG stress and inducible by exogenous ABA and GA3 hormone treatments. A MsBAN-GFP fusion protein expression vector used to analysis the subcellular location in protoplasts from *Arabidopsis* leaves, which showed that the GFP signal is localized in plastid. In order to further test the MsBAN gene expression, we sequenced a genomic DNA cloned including the gene promoter and functional domains. In addition to TATA-box, many elements also have been detected, such as ABA-response element: ABRE, GA-response element: GARE motif, MYB-binding domain, ERF-binding domain, and especial ten light-response elements. These elements and motifs suggested that MsBAN may be involved in many stress response regulation. The MsBAN was efficiently expressed and purified in *Escherichia coli* using the protein re-construction method. The BAN gene was over-expressed in *Arabidopsis*, and detectable by GUS staining. This work provides an experimental system for functional analysis of an important tannin synthesis-related gene, which will be useful to improve *Medicago sativa*.

## A High-resolution Method for the Localization of Proanthocyanidins in Plant Tissues

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Histochemical staining of plant tissues with 4-dimethylaminocinnamaldehyde (DMACA) or vanillin-HCl is widely used to characterize spatial patterns of proanthocyanidin accumulation in plant tissues. These methods are limited in their ability to allow high-resolution imaging of proanthocyanidin deposits. Tissue embedding techniques were used in combination with DMACA staining to analyze the accumulation of proanthocyanidins in *Lotus corniculatus* and *Trifolium repens* tissues. Embedding of plant tissues in LR White or paraffin matrices, with or without DMACA staining, preserved the physical integrity of the plant tissues, allowing high-resolution imaging that facilitated cell-specific localization of proanthocyanidins. A brown coloration was seen in proanthocyanidin-producing cells when plant tissues were embedded without DMACA staining and this was likely to have been due to non-enzymatic oxidation of proanthocyanidins and the formation of coloured semiquinones and quinones. This report presents a simple, high-resolution method for analysis of proanthocyanidin accumulation in organs, tissues and cells of two plant species with different patterns of proanthocyanidin accumulation, namely *Lotus corniculatus* (birdsfoot trefoil) and *Trifolium repens* (white clover). This technique was used to characterize cell type-specific patterns of proanthocyanidin accumulation in white clover flowers at different stages of development.



# Biosynthesis of Proanthocyanidins in White Clover Flowers: Cross Talk within the Flavonoid Pathway

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Proanthocyanidins and anthocyanins are produced by closely related branches of the flavonoid pathway and utilize the same metabolic intermediates. Previous studies have shown a flexible mechanism of flux diversion at the branch-point between the anthocyanin and proanthocyanidin pathways, but the molecular basis for this mechanism is poorly understood. Floral tissues in white clover (*Trifolium repens*) plants produce both proanthocyanidins and anthocyanins. This makes white clover amenable to studies of proanthocyanidin and anthocyanin biosynthesis and possible interactions within the flavonoid pathway. Results of this study show that the anthocyanin and proanthocyanidin pathways are spatially co-localized within epidermal cells of petals and temporally overlap in partially open flowers. A correlation between spatio-temporal patterns of anthocyanin and proanthocyanidin biosynthesis with expression profiles of putative flavonoid-related genes indicates that these pathways may recruit different isoforms of flavonoid biosynthetic enzymes. Furthermore, in transgenic white clover plants with down-regulated expression of the anthocyanidin reductase gene, levels of flavan 3-ols, anthocyanins, and flavonol glycosides and the expression levels of a range of genes encoding putative flavonoid biosynthetic enzymes and transcription factors were altered. This is consistent with the hypothesis that flux through the flavonoid pathway may be at least partially regulated by the availability of intermediates.

## Construction of *Medicago truncatula* Genetic Map by EST-SSR and QTL Analysis of Leaf Traits

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Our study aimed at identifying the multifoliate trait of alfalfa, and breeding novel alfalfa cultivar. *Medicago truncatula*, as a model legume, provided an important information sources for leguminous crops and pastures. This report has screened 425 pairs of SSR, which including 100 pairs of *Medicago truncatula* EST-SSR and 325 other pairs of *Medicago truncatula* SSRs. The 143 F<sub>8</sub> RIL population was constructed by the A17 and A20 lines of the French cultivar Jemalong. The genetic linkage map of *Medicago truncatula* was constructed with 129 markers. The map contained 16 groups, which accounted for 914cM of genome length of *Medicago truncatula*. The average distance of marker was 7.1 cM. The result of 129 markers showed 42 segregation distortion markers in the 8 groups. The QTL analysis show that six QTL distributed among in four linkage groups controlled leaf length, and their contribution rate is between 16.5% and 34.9%. Five QTL distributed in five linkage groups controlled leaf width, and their contribution rate was between 16.3% and 30.2%. There were six QTL which controlled the leaf length on LG1a, LG3, LG6a, and LG7c. LG1a had two QTL and the contribution rate was 30.7% and 34.9%. The QTL qLFL-1-2 had the highest LOD, which was 6.83. The QTL controlling leaf width were distributed among LG1b, LG2b, LG6a, LG6c, and LG7c. The contribution rate in LG1b was 30.2%, its additive effect size was -3.71, and its LOD size 3.69. In the 11 detected QTL, the additive effect size all came from the parent A20.

## Development of molecular marker resources for tall fescue

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Tall fescue [*Festuca arundinacea* Schreb. syn. *Lolium arundinaceum* (Schreb.) Darbysh] is an important cool season perennial hay and pasture grass in the temperate regions of the world. Attempts have been undertaken to develop molecular marker resources for the species. Parents (B400 and W279) of a mapping population were selected for this study. Total RNA was collected from root, stem, leaf and inflorescence and pooled for each parent. RNA-seq was carried out at the NCGR using Illumina platform. A total of 78.4 M and 55.6 M reads were obtained from the drought tolerant B400 and susceptible W279 parents, respectively. There were 54,295 transcripts produced from the B400, and 76,219 from the W279 data. SSR motifs were identified in 2,084 and 1,646 of the assembled sequences in B400 and W279, respectively. In silico alignment identified polymorphic SSR loci between the two parents. SNPs homozygous in B400 and heterozygous in W279 and vice-versa were identified. Sequences containing these SNPs were blast against the Brachypodium genome and SNPs evenly distributed throughout the genome were selected. In the first phase 96 primer pairs have been developed and tested using high resolution melting analysis. All primers successfully amplified in two parents between Tm gradients from 56 to 72°C and 78 of them showed single peak candidates. Prescreening in parents and a subset of the population identified 36 primers suitable for mapping analysis, 28 were with complex patterns, and 14 were polymorphic between parents but monomorphic in the population. Genotyping and mapping of the 36 selected SNPs are in progress.

## Gene Discovery and Molecular Marker Development Based on High-Throughput Transcriptome Sequencing in *Brachiaria brizantha* Hochst ex A. Rich.

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*Brachiaria* is an economically-important forage grass genus in Central and South America. Productivity per hectare and weight gain per head are both significantly higher in cattle grazed on *Brachiaria* pastures compared with those grazing native savana. *B. brizantha* Hochst ex A. Rich is an apomictic tetraploid species, which does also have a diploid sexual form that is not currently used in agricultural systems. The *B. brizantha* cv. 'Marandu' has been widely adopted due to its resistance to spittlebug as well as displaying good persistence under grazing and resistance to water deficit. However, *B. brizantha* still has opportunities for improvement in digestibility and nutritive value. The species would benefit significantly from an enabling molecular breeding program for varietal development. To generate initial resources a pooled cDNA library was prepared from RNA samples from different tissues and subsequently sequenced using GS FLX Titanium technology. A total of 445,357 sequence reads were obtained, corresponding to c. 130 Mbp, which were assembled using Newbler software v 2.3, generating 12,005 contigs of a combined length of 6,373,398 nucleotides. The transcript sequence contigs generated were also analysed for the presence of simple sequence repeats (SSRs). A total of 1,699 SSR motifs were identified and primer pairs were designed for SSR amplification for 1,446 contigs using the batch Primer3 software. This molecular marker resource will enable molecular breeding efforts in *Brachiaria* species.

# Gene Discovery and Molecular Marker Development Based on High-Throughput Transcriptome Sequencing in *Paspalum dilatatum* Poir.

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Dallisgrass (*Paspalum dilatatum* Poir.) is a highly productive C<sub>4</sub> grass with a wide distribution within temperate-warm regions and has a growing season from late spring to late summer. It is a native grass species of South America, with special relevance for dairy and red meat production. The forage quality of *Paspalum* is higher than other C<sub>4</sub> forage species and the species exhibits other desirable traits such as frost tolerance and tolerance of water stresses. However, there are still significant opportunities for molecular breeding to improve the productivity of this species. With the ongoing reductions in cost and resources required to deliver genomic tools to underpin molecular breeding strategies, opportunities now arise for many species to rapidly benefit from these developments. A cDNA library was prepared from pooled RNA of different tissues (stems, roots, leaves and inflorescences) from the final reproductive stage of *P. dilatatum* 'Primo' and subsequently sequenced using GS FLX Titanium technology. A total of 324,695 sequence reads were obtained, corresponding to c. 102 Mbp, which were assembled using Newbler software, generating 20,169 contigs of a combined length of 9,336,138 nucleotides. The generated contigs were subjected to BLAST sequence analysis against the full genome sequences of *Oryza sativa* subsp. japonica, *Sorghum bicolor* and *Brachypodium distachyon* as well as against the UniRef 90 protein database. The transcript sequence contigs generated were also analysed for the presence of simple sequence repeats (SSRs). A total of 2,339 SSR motifs were identified and primer pairs were designed for SSR amplification for 1,989 contigs using the batch Primer3 software. Empirical validation of a cohort of 96 SSRs has been performed, with acceptable performance confirmed for 64% of the markers tested. This molecular marker resource will enable molecular breeding efforts in *Paspalum* species.

## Genetic engineering for the improvement of forage digestibility in warm-season grasses

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Warm-season grasses have relatively low nutritive value and poor digestibility in comparison to most temperate grasses and other forages. It is believed that the low digestibility of warm-season grasses is due to its high lignin content. Cinnamyl alcohol dehydrogenase (CAD) is a key enzyme involved in lignin biosynthesis and down regulation of CAD gene expression may be an effective way of improving forage digestibility. Bahiagrass (*Paspalum notatum* Flüggé) plants were transformed using antisense and RNAi vectors containing the sorghum (*Sorghum bicolor* (L.) Moench) CAD gene. These transgenic plants showed decreased lignin content and 4 out of 9 lines showed higher level of in vitro-digestibility than seed-derived control plants. Transgenic lines plants showed reduced size and lower dry matter production compared with control plants. However, one line was indicated the opposite phenotype with higher dry matter production and 6.9% greater in vitro-digestibility compared to control plants. This study demonstrated that genetic engineering has the potential to improve warm-season forage grass digestibility.

# Genetic Variation, Population Structure and Linkage Disequilibrium in Populations of Perennial Ryegrass Selected for Freezing Tolerance

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Freezing tolerance is a trait of major agronomical importance in northern and central Europe. The plant material studied was derived from an experimental population established from pair-crosses and polycrosses among five genotypes of the *Lolium* Test Set (genotypes LTS 3, 4, 11, 15, and 16), employed in the EU project GRASP to represent diverse trait performance and climatic adaptation in Europe. Three-hundred individual plants from Syn-0 generation were first screened for freezing tolerance. Then two generations of differential selection (selection intensity 10%) using artificial freezing tests followed by open pollination of the 30 selected genotypes in isolation was conducted generating a high-freezing tolerant (HFT-2) and a low-freezing tolerant (LFT-2) population. Every round of selection was performed using 300 plants from each divergent population. An unselected Syn-2 generation (US) was generated by intercrossed 100 randomly selected individuals every generation to quantify the effect of random genetic drift. A total of 80 individuals (HFT-2: 24, LFT-2: 29 and US: 27) were genotyped using 278 genic *Lolium perenne* SNPs distributed across the linkage groups as follows: LG1 (37); LG2 (57); LG3 (32); LG4 (40); LG5 (39); LG6 (37); and LG7 (36). The SNP genotyping was performed using the Sequenom MassARRAY iPLEX Gold technology. Linkage disequilibrium (LD) was measured within each chromosome among the three frost tolerant populations and observed higher LD in the selected populations than in the unselected. LD ( $r^2$ ) decay varied among the three populations especially for LG5 and LG7, linkage groups that contain several QTLs/genes for freezing tolerance. Structure analysis further identified subpopulations within the selections. Data for allele frequencies, genetic diversity, and polymorphic information content will be presented and discussed.

## Isolation and functional study of MsBAN gene in *Medicago sativa*

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Forages with high digestibility and abundant soluble proteins can cause pasture bloat, the condensed tannins can reduce the incidence and severity of bloat for ruminant livestock. The BANYULS (BAN) gene is a key enzyme in condensed tannin synthesis pathway. A cDNA of BAN gene is isolated from *Medicago sativa* by using RACE method. Some conserved motifs were detected including a NADP binding site (VIGGTGFVASLLIKQLLEKGY) in the N-terminal region. Southern blot was performed and identified the single-copy of MsBAN gene in the genome of *M. sativa*. The results of Real-time PCR indicated that the expression level of BAN gene is highest in pods, and least in flower. The expression of BAN gene is up-regulated by NaCl and PEG stress. Under exogenous hormones ABA and GA3 treatments, the gene expression was also significantly induced. We constructed the vector of MsBAN-GFP fusion protein to analysis the subcellular location in protoplasts from *Arabidopsis* leaves, and found that the GFP signal is localized in plastid. In order to further verifying the MsBAN gene expression, we cloned the full length of gene promoter and analysis the domains. In addition to TATA-box, many elements also have been detected, such as ABA-response element: ABRE, GA-response element: GARE motif, MYB-binding domain, ERF-binding domain, and especial ten light-response elements. These elements and motifs suggested that MsBAN may be involved in many stress response regulation. By using protein re-construction method, the prokaryotic express of MsBAN protein was studied. The MsBAN could be highly purified and efficiently expressed in *Escherichia coli*. We also constructed over-expression vector, and transformed the *Arabidopsis* plants. The positive lines identified using GUS staining was kept for further study. These works provided a candidate gene for forage quality improvement and found a base for further functional study of tannin synthesis-related gene.

# Methanogenic potential in the rumen and quantitative trait locus analysis of subterranean clover

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Subterranean clover (*Trifolium subterraneum* L.) is one of the important forage pasture legumes in ruminant production. While it has good agronomic and nutritive properties, consumption of forages by the ruminant is readily associated with methane production during microbial fermentation in the gut ('methanogenic potential'). To date, 42 cultivars of subterranean clover have been released in Australia, but further genetic gains can be expected if plant breeders are able to exploit new traits, and one such trait is plant methanogenic potential. In our preliminary studies, we have found that cultivars of subterranean clover vary in their methanogenic potential, with significant differences observed between two cultivars, i.e. Daliak and Woogenellup. The aim of the current study was to examine a bi-parental cross (F<sub>2</sub> population) between these two cultivars in order to identify regions of the genome associated with variation in methanogenic potential and identify candidate genes for marker assisted breeding in future. Briefly, plant samples were fermented using an in-vitro batch fermentation tests and methane was measured in these treatments. This was followed by a QTL analyses for this trait. We have found that there was (significant P<0.05) variability in methanogenic potential amongst the samples tested. The molecular-genetic information generated is expected to provide a platform to develop subterranean clover cultivars with lower methanogenic potential in the rumen, which will offer a powerful new approach to reduce greenhouse gas emissions from ruminants.

## Methodologies for marker assisted selection in forage breeding schemes

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Marker assisted selection (MAS) on forage species is still rarely used by breeders except for the introgression of simple traits. This could be due to the lack of cheap and high throughput markers and / or of methodologies to apply MAS in their breeding material. Due to inbreeding depression, forage breeding material generally comes from intercrosses of several selected plants. The population resulting from an intercross consists in several full-sib families linked by the parents of the intercross, equivalent to a multipopulation connected design. The objective of this study was to test methodologies to detect Quantitative Trait Loci (QTL) in multipopulation connected designs using plant height of perennial ryegrass as an experimental system. In a first experiment, based on three large biparental populations (200 plants each) connected by a common parent, the multipopulation connected analysis from MCQTL software was found to be more powerful for the identification of favorable alleles than the detection within each population with QTL Cartographer software. Numerous QTL were detected in spite of a narrow genetic basis of the studied material. In a second experiment, the same analysis was carried out to detect QTL directly in breeding material: a population (252 plants) resulting from the intercrosses of six selected plants. Molecular markers were used to identify the male parent and confirm the female parent of each sibling. Full-sib family sizes varied from 2 to 28 individuals. A few QTL were detected with the multipopulation connected analysis but a better equilibrium in the family sizes would improve the efficiency of the design. This study gave new insights on the use molecular markers in complex forage breeding schemes that may be tractable using new high throughput genotyping techniques.

## Overexpression of Alfalfa Mitochondrial Heat Shock Proteins23 Confers Enhanced Tolerance to Oxidative Stresses

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The role of heat shock proteins (HSPs) in protecting cells against damage associated with environmental stresses has been well documented in a wide range of organisms, ranging from human to bacteria. As an important organelle in plant cells, mitochondria perform many essential functions. Mitochondrial structural breakdown and abnormal metabolic activity affects cell viability at high temperature. Therefore, the capacity of mitochondria to tolerate high temperature greatly impacts the activity and survival of the entire cell. Mitochondrial sHSPs (mHSPs) are synthesized in the cytoplasm as precursors and then transported into the mitochondria. The cloning and characterization of a gene (mHSP23) coding for a heat shock protein in alfalfa in a prokaryotic and model plant system is described. mHSP23 contains a 633 bp ORF encoding a polypeptide of 213 amino acids and exhibits greater sequence similarity to mitochondrial sHSPs from dicotyledons than to those from monocotyledons. When expressed in bacteria, recombinant mHSP23 conferred tolerance to salinity and arsenic stress. Furthermore, mHSP23 was cloned in a plant expressing vector and transformed into tobacco, a eukaryotic model organism. The transgenic plants exhibited enhanced tolerance to salinity and arsenic stress under ex vitro conditions. In comparison to wild type plants, transgenic plants were exhibited significantly lower electrolyte leakage. Moreover, the transgenic plants had superior germination rates when placed on medium containing arsenic. Taken together, these overexpression results imply that mHSP23 plays an important role in salinity and arsenic stress tolerance in transgenic tobacco. This approach could be useful to develop stress-tolerant plants including forage crops.

## Searching in sequences of Leymus BAC clones for genes controlling salt tolerance

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Many species of *Thinopyrum* and *Leymus* are known to be highly salt tolerant. Salinity tolerance in diploid *Thinopyrum elongatum*, thus all polyploid *Thinopyrum* species too, is controlled by genes on different chromosomes. Some candidate genes, including genes for peroxidase precursor, for salinity tolerance had been identified through a microarray study on two wheat translocation lines involving chromatins from *Thinopyrum junceum* and *Aegilops speltoides*. In addition to toxic effects, salt and heavy-metal stresses can also induce oxidative stress with the formation and accumulation of reactive oxygen species (ROS). Antioxidant enzyme activities of superoxidase dismutase, catalase, peroxidase, ascorbate peroxidase and glutathione reductase appeared to be responsible for the higher tolerance to abiotic stresses in plants. Bacterial artificial chromosome (BAC) genomic DNA library representing about 6.1 haploid genome equivalents of tetraploid *Leymus* had been previously developed. By screening the entire BAC library with designed Overgo primers, 11 BAC clones that might contain homoeologous genes controlling salinity tolerance or being affected by salt stress had subsequently been selected and sequenced. Contig sequences of two BAC clones targeting the peroxidase precursor will be analyzed to find polymorphisms among the orthologous genes from various plant species.

# Sequencing and annotation of the perennial ryegrass mitochondrial genome

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Perennial ryegrass (*Lolium perenne* L.) is one of the most important forage and turf grass species adapted to temperate regions worldwide. As an allogamous species, perennial ryegrass is currently being improved as populations, thereby not fully exploiting the potential of heterosis. The mitochondrial genome of perennial ryegrass was sequenced as part of a project with the long term goal to control pollination for the development of hybrid breeding schemes using cytoplasmic male sterility. Intact mitochondria were isolated from leaves of a four-month-old plant and used for DNA extraction. Mitochondrial DNA was sequenced using the 454 GS-FLX sequencer, resulting in 287,367 single reads with an average read length of 406bp. The reads were quality filtered and contaminating chloroplast sequences were removed from the dataset. De novo assembly using the CLC Genomics Workbench resulted in approximately 2,400 contigs, of which nine contigs ranging from 6,735 to 219,170 bp were identified as mitochondrial sequences. The scaffold information from a draft assembly of the perennial ryegrass genome, which became available in our laboratory, was used to order and orientate the contigs. Primers were designed to PCR amplify across gaps. The resulting fragments were sequenced and used to close gaps between established contigs. Finally, the mitochondrial genome was assembled into a master circle molecule of 678,559 bp. We are currently working on the annotation of the mitochondrial genome and have already identified large duplications in the entire genome, as well as major rearrangements compared to mitochondrial genomes of other grass species. The genetic structure of the mitochondrial genome of perennial ryegrass will be presented. Our work constitutes a solid basis for comparative analyses of mitochondrial genomes within *Lolium* and between closely related grass species, and will – on the long run – increase our understanding of cytoplasmic male sterility in perennial ryegrass.

# Sucrose starvation up-regulated the expression of LcSUT1 in leaf sheath of *Leymus chinensis* under defoliation

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*Leymus chinensis* is a perennial grass of Gramineae. It is widely distributed in the northern China and the Republic of Mongolia. The high vegetative productivity, protein content and palatability of *L. chinensis* makes it a key species in the rangeland. Grazing or cutting is removal of grass leaves by animal or artificial harvesting, which lead to the loss of the photosynthetic organs and therefore reallocation of carbohydrate in whole plants. Sucrose represents one of the major products of photosynthesis. It is synthesized in source organs and translocated to sink organs to support their growth. Although defoliation in *Lolium perenne* led to the significant increased expression of LpSUT1 in leaf sheath, the induction mechanism was not clear. To better understand the function of SUT1 after defoliation, we cloned a sucrose transporter LcSUT1 from *L. chinensis*. Heterologous expression of LcSUT1 in yeast proved that it was a functional sucrose transporter. Tissue-specific expression analysis showed that LcSUT1 highly expressed in leaf and leaf sheath, which suggested LcSUT1 may play important roles in these tissues. Defoliation removal of the leaf blade that functions as source tissue to perform photosynthesis and fix carbon, certainly will lead the carbon reallocation in the whole plants. Our results showed that the LcSUT1 was significantly up-regulated in leaf sheath after defoliation, but not induced by wounding. The expression level of LcSUT1 was also increased when callus, a model of sink tissue, grew on N6 medium without sucrose. Our hypothetic conclusion is that the increased expression of LcSUT1 in leaf sheaths after defoliation may be the results of sucrose starvation.



## ***Biserrula pelecinus*: genome, phenome and metabolome analyses**

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*Biserrula pelecinus* L. is a Mediterranean annual pasture legume which performs best on well-drained sandy loams and medium loams with a pH 4.5 – 7. Diversity analysis of a germplasm collection containing 279 accessions using 18 agro-morphological traits, 22 eco-geographical datasets from the collection sites, and amplified fragment length polymorphism (AFLP) markers was conducted to develop a core collection constituting c. 10% of the original collection. This core collection adequately represented the diversity of the whole collection. A previous comparison, chiefly between pasture legume species, revealed significant variation for in vitro methane production, *Biserrula pelecinus* showed particularly low methanogenic potential. It has been proposed that the core collection of *B. pelecinus* may exhibit variation for fermentability traits. The *Biserrula* core, comprising 30 accessions from seven different countries, in addition to subterranean clover and bladder clover control samples, was examined for variability of in vitro rumen fermentation, including methane production by rumen microbes, and possible association of these traits to variation for plant morphological characters. Significant variability in fermentability profiles was revealed between accessions. The methanogenic potential of the accessions showed a 90% broad-sense heritability value. Although all of the tested accessions showed low gas pressure and methanogenic potential, they also sustained volatile fatty acid (VFA), which indicates low digestibility and high nutritive value. Metabolomic profiles of five high and five low methanogenesis accessions were developed, and correlations between metabolites and methanogenic potential were investigated. These correlations may be used for selection of an environmentally friendly cultivar of *B. pelecinus* in the near future.

## **Determination of genetic components of eight half-sib family populations of alfalfa in central valley of Mexico**

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Genetic variance estimators in forage crops are essential parameters to determine the response to selection for agronomic and quality traits; and these estimators are influenced by the breeding scheme, environment and population background. With this aim, we estimated the genetic components of eight alfalfa populations (Julia, San Miguelito, Macate, Mediterranea, Atlixsco, Tanverde, Jupiter and INI-76) using a Half-Sib Family design (10 HFF per population) at two locations in the Central Valley of Mexico. A lattice design (8x10) with three replications (12m<sup>2</sup> per plot) and seven harvests in 2010 was used. Phylogenetic relationship among and within populations was estimated by AMOVA using 10 AFLP polymorphic primers combinations. 357 polymorphic amplicons resulted in 83.5% of molecular variance within populations and 16.5% among populations (GS=0.31 to 0.76), without clustering observed, indicating a strong genetic relationship for these populations. Association between molecular data and genetic components was verified, with total yield, stem/leaf biomass, height, stem number, and crude protein representing up to 54% of the genetic variance within populations, as shown in molecular results also. Additive genetic variance accounted up to 14% of genetic variance and heritability was 0.05, 0.08, 0.12 and 0.18 for total yield, stem yield, height, and leaf yield. E and GxE variance represented up to 79% of the phenotypic variance. NDF, ADF and CP showed intermediate narrow sense heritability (0.17, 0.19, 0.25, resp), assuming an additive effect for these traits. Expected genetic gain per selection cycle in total yield, crude protein, and stem number and yield was greater from Half Sib family than progeny test; however no differences was observed for leaf biomass, NDF, ADF, and plant height.

# Development and characterization of genomic simple sequence repeat markers in *Cynodon transvaalensis*

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*Cynodon transvaalensis*, commonly called 'African bermudagrass', has economic value as a turf grass, and significant breeding value to hybridize with *C. dactylon* var. *dactylon* to develop new interspecific clonal turf cultivars. Simple sequence repeat (SSR) markers are one of the most widely used co-dominant molecular marker systems, which should be highly suitable for genetic investigations in highly heterozygous African bermudagrass. However, few SSR markers are available in *C. transvaalensis*. Accordingly, the objective of this study was to develop genomic SSR markers in the species. Genomic DNA of 'TN 4200 24-2' *C. transvaalensis* accession was used to construct four SSR genomic libraries enriched with CA, GA, AAG, AAT as core sequences. The SSR-enriched DNA inserts sequenced at Oklahoma State University Core Facility generated 3050 trimmed sequences, which were examined with CAP3 program to eliminate the redundancy and blast against the previously developed SSR markers. Consequently, 1426 non-redundant primer pairs were designed from the sequences with SSR locator software. After the initial screening on 'TN 4200 24-2' genotype, 981 primer pairs were effective in amplifying reproducible clear target bands. A panel of another seven *transvaalensis* accessions plus the DNA donor genotype was utilized to test polymorphisms of the 981 SSR markers. An inheritance study was conducted in six *transvaalensis* progeny plants with two parent plants 'Uganda' and 'T577' accessions. The laboratory work of testing SSR markers polymorphism and inheritance was just finished, and more results will be updated.

## Development, characterization, and cross-taxon utility of EST-derived SSR markers in alfalfa

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Due to their highly polymorphic and co-dominant nature, SSR markers are a common choice for assaying genetic diversity and genetic mapping. EST- derived microsatellite markers identified from publicly available sequence databases may serve as time effective and less costly alternative markers in alfalfa. By analysis of 12,371 M. sativa EST sequences from the NCBI, 774 SSR containing ESTs were identified corresponding to one SSR per 7.7 kb of EST sequences. Tri-nucleotide repeats (48.8%) were the most abundant followed by di- (26.1%), tetra- (11.5%), penta- (9.7%), and hexanucleotide (3.9%) motifs. The top five motifs included AG/CT repeat (17.2%) followed by AAG/CTT (15.1%), ACC/GGT repeat (7.2%), ATC/ATG repeat (7.1%), and AAC/GTT repeat (6.9%). One hundred primer pairs were successfully designed and used for validation of the amplification and assessment of the polymorphism among 28 alfalfa genotypes representing six *M. sativa* subspecies. Nearly, 56 primer pairs produced SSR bands of expected size length in all *Medicago* spp, of which 25 primer pairs exhibited polymorphic bands among the 28 genotypes. The PIC values ranged from 0.62 to 0.94 with the mean of 0.86, indicating a high level of informativeness within these EST-SSRs. The high level of marker transferability across other *Medicago* species was 100% in *M. lupulina*, 91.1% in *M. minama*, and 47.0% transferability across genera in *Trifolium repens* and *Melilotus albus*. These EST-SSRs in alfalfa will be a valuable resource for future genetic studies, like construction of linkage maps, diversity analysis, quantitative trait locus/association mapping, and molecular breeding of alfalfa.

# Differentiation of perennial and Italian ryegrasses at both species- and variety-specific levels using multiplexed SNP Markers

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The *Lolium* species *L. perenne* (perennial ryegrass) and *L. multiflorum* (Italian ryegrass) are widely cultivated as forage grasses and they are closely related, such that natural and artificial hybridisation produces fully fertile F<sub>1</sub> progeny. Cultivars from both species and interspecies hybrid (*L. x. boucheanum*) are represented in the current pasture seed market. Differentiation at both the species- and varietal levels are not readily undertaken by morphological comparison and enzyme electrophoresis. The objective of this study was to differentiate *L. perenne* and *L. multiflorum* at both species and cultivar levels by discovery and implementation of multiplexed single nucleotide polymorphisms (SNP) marker genotyping assays. Genomic amplicon sequencing from *L. perenne* permitted SNP prediction and validation to allow assembly of a 384-plex Illumina GoldenGate™ assay panel. The panel was used to genotype plants from cultivars of each non-hybrid and hybrid taxon. High transferability of SNP marker performance was observed between *L. perenne* and *L. multiflorum*. Neighbor-joining (NJ) tree analysis based on pair-wise genetic distances between cultivars revealed that clustering of perennial and Italian ryegrass varieties into two distinct groups, with intermediate positioning of the hybrid variety. At the varietal level within species, the NJ tree generally reflected breeding history and pedigree. Tetraploid varieties could also be distinguished from diploid types based on SNP data, which exhibited 5 and 3 distinct genotypic classes, respectively. These results demonstrated that multiplexed SNP markers could differentiate *L. perenne* and *L. multiflorum* at both the species and variety-specific levels. The primary catalogue of current varieties from both species will provide valuable information for cultivar choice on farm and direction for future varietal development in breeding.

## Genetic and geographical differentiation of two hexaploid perennial Triticeae grasses in China (Poaceae)

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*Elymus nutans* and *Roegneria kamoji* are two important and widely distributed perennial Triticeae forage grass species in China. These species also possess the same genome constitution (2n=6x=42). Here, morphology, gliadin and molecular markers have been used to evaluate the genetic and geographical divergence of 63 *E. nutans* accessions from West China and 40 *R. kamoji* accessions from Southwest China. Substantial genetic diversity was identified between the two species. The average Shannon-Weaver index of *E. nutans* and *R. kamoji* based on 30 quantitative characters was 2.21 and 1.96, respectively. Within *E. nutans*, a total of 42 bands were detected, 90.48% of which were polymorphism. The Nei's genetic similarity (GS) coefficient ranged from 0.32 to 1.00 with an average of 0.63. Accordingly, among *R. kamoji*, there were 29 bands with a polymorphism rate of 89.66% and the genetic similarity coefficients ranged 0.45-1.00 with an average of 0.70. Moreover, different molecular markers (SSR, SRAP, RAPD) were used in *E. nutans*, which revealed that the GS ranged 0.19-1.00 with an average of 0.70. The results of cluster analysis were congruous with the gliadin, which indicated that the results of cluster were related with the geographical origin. Furthermore, the distinct geographical differentiation was also revealed in the two species. These results also showed that ecological factors, such as altitude, climate, and landform, played a crucial role in the differentiation. The Qinghai-Tibet Plateau and Southwest China might be the diversification center for *E. nutans* and *R. kamoji*, respectively.

## Genetic diversity of *Miscanthus sinensis* based on simple sequence repeats (SSR) markers

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*Miscanthus sinensis* is a C<sub>4</sub> perennial grass, native to most parts of China. Since *M. sinensis* has high biomass yield and is well adapted to marginal lands, it has been selected as a candidate for bioenergy resources. In order to explore genetic diversity of wild population *M. Sinensis* in Sichuan, China, 103 wild samples were collected and analyzed by simple sequence repeats (SSR) markers. Ten pairs of primers designed from the EST-SSR markers of sorghum (Wang, Barkley 2005) were used following the touchdown PCR program to amplify the *M. sinensis* genome, and the data was analyzed by NtSys-2 and PopGen32. The results showed that a total of 163 fragments were amplified, of which 156 were polymorphic, that was 95.71%. The average Nei's gene diversity was 0.36 and mean Shannon index was 0.53. It shows that the provenance of *Miscanthus* plants have high genetic diversity in Sichuan, China. The genetic similarity (GS) among all accessions ranged from 0.42 to 0.95, Cluster analysis of UPGMA showed that 103 samples could be classified into 6 groups at the level of GS 0.63, and the genetic distance between these sample were 0.03 to 0.04. But the result of clustering and the germplasm collecting locations were not correlated.

## Genetic variation of alfalfa traits related to competition in alfalfa-fescue mixtures

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Alfalfa is an important forage crop with low input costs related to atmospheric nitrogen fixation. Mixtures of alfalfa with grass species combine, in a single sward, the advantages of the two species: seasonal growth pattern, biochemical composition, nitrogen fixation or absorption. The survival of each species results from the ability of the plants to get the resources needed for its growth among which light radiation and nitrogen are of major importance. The present study aims at describing genetic variation for traits related to competition in alfalfa-fescue mixture. Forty six contrasting genotypes from ten alfalfa varieties and two genotypes (a forage type and a turf type) of tall fescue were planted on September 2010. An innovative design was used: for each pair of alfalfa-fescue genotypes, it included spaced plants, monocultures in dense conditions (plots of 7 plants, the target plant being surrounded by 6 plants, 7 cm apart) and alfalfa-fescue mixtures (plots of 3 alfalfa and 4 fescue plants, 7 cm apart), with 3 repetitions. Each genotype of alfalfa and the two genotypes of fescue were clonally propagated to establish the treatments. Four harvests were taken in 2011; dry matter biomasses were recorded for the target plant and the surrounding plants. In analyses of variance, the effects of alfalfa genotypes and treatments on biomass production were highly significant. The interaction between genotypes and treatments was only significant in the fourth cut, showing that the differences among alfalfa genotypes for biomass production depended on the surrounding plants. Traits related to plant height, nitrogen content, growth habit were also recorded. This design will be studied again in 2012. A similar design was established with the F<sub>1</sub> progeny of mapping populations from contrasting clones of the initial population, in order to analyze genetic control of traits responsible for competition for light or nitrogen.

# Genome-wide SNP identification in multiple morphotypes of allohexaploid tall fescue (*Festuca arundinacea* Schreb.)

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As an outbreeding allohexaploid species, the agronomically important pasture grass tall fescue (*Festuca arundinacea* Schreb. syn. *Lolium arundinaceum* (Schreb.) Darbysh.) provides an example of a plant species with a complex genome. Furthermore, three distinct morphotypes of tall fescue have been identified (Continental, Mediterranean and rhizomatous) which differ with respect to geographical origin, growth patterns and morphological features. A bioinformatic pipeline was developed that successfully identified SNPs within genotypes from distinct tall fescue morphotypes, following the sequencing of 414 polymerase chain reaction – generated amplicons using GS FLX Titanium technology. Equivalent amplicon sets were derived from representative genotypes of each morphotype, including six Continental, five Mediterranean and one rhizomatous. A total of 8,584 and 2,292 SNPs were identified with high confidence within the Continental and Mediterranean morphotypes respectively. The success of the bioinformatic approach was demonstrated through validation (at a rate of 70%) of a subset of SNPs using both SNaPshot<sup>TM</sup> and GoldenGate<sup>TM</sup> assay chemistries. Furthermore, the quantitative genotyping capability of the GoldenGate<sup>TM</sup> assay revealed that approximately 30% of the putative SNPs were accessible to co-dominant scoring, despite the hexaploid genome structure. The sub-genome-specific origin of each SNP validated from Continental tall fescue was predicted using a phylogenetic approach based on comparison with orthologous sequences from predicted progenitor species. This SNP collection may now be refined and used in applications such as cultivar identification, genetic linkage map construction, genome-wide association studies and genomic selection in tall fescue.

## Marker-Trait Association of Rangeland and Turf Traits in Hybrids of *Festuca idahoensis* and *Festuca ovina*

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The fine fescue species *Festuca idahoensis* Elmer is native to the Intermountain West and has potential for low-input turf and rangeland applications. The poor seed production and weak seedling vigor of this species may be overcome by identification and improvement of interspecific hybrids between *F. idahoensis* and *F. ovina* L. concomitant with detection of economically important marker-trait associations for plant development. To this end, we assessed a population of cross-progenies derived from an open-pollinated crossing block of *F. idahoensis* and *F. ovina* parents for morphological characteristics and AFLP markers. A total of 261 cross progenies were cloned and planted in a replicated trial at Hyde Park, UT., with seven *F. idahoensis* and three *F. ovina* commercial controls. Plant vigor, total biomass, height, width, re-growth, 100 seed weight, and seed production were evaluated in 2010 and 2011. Cross-progenies were significantly different from each other and controls ( $P < 0.0001$ ) for all phenotypic traits over both years. Plant vigor was significantly correlated ( $P < 0.0001$ ) with plant height ( $r^2 = 0.91$ ), width ( $r^2 = 0.83$ ), and total biomass ( $r^2 = 0.89$ ). Most of the cross-progenies grouped together while the controls were unique based on Principal Component Analysis (PCA) of the morphological data. This result was supported by genetic differentiation analyses of six selective AFLP primer combinations which provided a neighbor-joining tree and Bayesian clustering by STRUCTURE. AFLP results showed the interspecific nature of the cross-progenies while the controls grouped together by species. Single-factor ANOVA of the AFLP markers with the morphological data revealed three markers that were associated with height and 18 other markers associated with multiple traits over both years. These results provide a starting point for improvement of *F. idahoensis* with traits from *F. ovina* using a combination of phenotypic and marker-assisted selection.

# Molecular Characterisation and Analysis of Genetic Diversity within a Globally Distributed Collection of Tall Fescue (*Festuca arundinacea* Schreb.).

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Tall fescue (*Festuca arundinacea* Schreb. syn. *Lolium arundinaceum* (Schreb.) Darbysh.), is an outbreeding allohexaploid ( $2n = 6x = 42$ ) cultivated for pasture worldwide. The majority of tall fescue cultivars are classified as Continental types, and have arisen from northern European ecotypes that display maximum growth over summer periods. All additional cultivars can generally be categorized as either Mediterranean or rhizomatous, which differ from Continental cultivars with respect to geographical origin, growth patterns and morphological features. Previous sequencing of a chloroplast genome-derived gene and the internal transcribed spacer region of ribosomal DNA from each of the three tall fescue morphotypes and related tall fescue cytotypes, has identified diagnostic nucleotides capable of characterising tall fescue germplasm. This approach has been used to screen a large collection of tall fescue and meadow fescue accessions held and maintained by the United States Department of Agriculture through the Germplasm Resource Information Network. Results have identified the presence of the three tall fescue morphotypes as well as tall fescue sub-species of varying ploidy levels in both collections. Characterisation of these accessions has enabled biogeographical analysis of each tall fescue morphotype and sub-species and as expected, has revealed a North African distribution of Mediterranean tall fescue and a wider European distribution of Continental tall fescue from Spain through to western China. Genetic structure within Continental, Mediterranean and rhizomatous tall fescue was investigated further through the amplification of 34 SSR markers. This analysis supports the divergence between Continental and Mediterranean tall fescue and also identifies subpopulations within the Continental germplasm that reflect morphological differences and geographical distribution. This work has provided an insight into the phylogeographical history of tall fescue whilst comprehensively characterising and dissecting a complex germplasm resource for the benefit of plant breeding.

## Molecular characterization of *Lotus tenuis* with contrasting behavior for salinity

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In Argentina over the past years there was a strong increase of agricultural production displacing livestock production in marginal areas. One of the species more relevant to these environments is *Lotus tenuis* Waldst. et Kit, perennial legume, diploid  $2n = 2x = 12$ , allogamous, with a spring-summer-autumn production. This species is naturalized in the fields of Flooding Pampa and valued for its contribution to forage supply of livestock systems in the region. The characterization of genetic variability of *L. tenuis* has been based on the description of morpho-physiological traits. The aim of this work was to conduct molecular characterization of two synthetic materials of *L. tenuis*, with contrasting behavior for salinity (tolerant vs. susceptible) according to preliminary research. Four SSR markers from *L. japonicus* were used to determine the presence/absence of the bands/alleles in 30 seedlings (six bulks each with five seedlings) from each synthetic material. There were 27% more present alleles in susceptible bulks than the tolerant ones. These results provided not only basic knowledge on the transfer of SSR from *L. japonicus* to *L. tenuis*, but also allowed the integration of molecular markers in breeding programs of the species and the initial identification of markers associated with salinity tolerance.

# Morphological Appraisal of *Festuca valesiaca* for Plant Improvement and Its Relatedness to the *Festuca ovina* Complex

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Fine-leaved *Festuca valesiaca* possesses varied environmental tolerance. However, its agronomic performance in the western United States and its genetic relationship to species of the *Festuca ovina* complex have not been investigated. Therefore, a project was designed to identify *F. valesiaca* accessions possessing high biomass production and seed yield to initiate breeding for low-maintenance growing environments and to examine their relatedness to the *F. ovina* complex. Twelve *F. valesiaca* accessions originating from Kyrgyzstan and eight commercial cultivars were transplanted to a field nursery at Blue Creek, Utah in a randomized complete block design (RCBD) with six replications. Plant height, plant width, total biomass, seed weight, and seed number per plant were evaluated between 2009 to 2011. Amplified fragment length polymorphisms (AFLP) with 10 primer combinations were used to evaluate the genetic relatedness between *F. valesiaca* and species of the *F. ovina* complex. Morphological trait evaluation indicated that plant height, plant width, and total biomass of the *F. valesiaca* accessions examined were equal to the control 'Cascade'. In contrast, the plant vigor and seed weight of accessions 'KGZ-094', 'KGZ-119', 'KGZ-229', and 'KGZ-242' were significantly higher than 'Cascade'. Principal component analysis using all traits as loading factors suggested that these accessions were distinct from the majority of the accessions examined. AFLP-based neighbor-joining cluster analysis defined five distinct groups consisting of diverse *Festuca* species (Outgroups; Group 1), *F. idahoensis* (Group 2), *F. rubra* (Group 3), *F. ovina* and *F. valesiaca* (Group 4), and *F. trachyphylla* (Group 5). This relationship of these species were further confirmed by an AFLP-based genetic structure analysis. In conclusion, *F. valesiaca* is closely related to *F. ovina*. Given their morphological attributes, *F. valesiaca* accessions 'KGZ-094', 'KGZ-229', and 'KGZ-242' should be considered for low-maintenance applications and use in plant improvement.

## Mutation Induction of Sorghum (*Sorghum bicolor*) by Gamma-Ray Irradiations

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Gamma ray irradiation is one of the most powerful tools for induction of mutations for genome analysis and gene isolation, as well as development of new varieties. Recent reports indicate that gamma-ray can induce both deletions with 1-5 bp and 10 to 90 thousand bp, and large deletions can knockout a tandem array in one time. The mechanisms of low glutelin contents of mutants are elucidated that the different size and the position of deletions generate different characteristics of mutations, such as act as dominant gene or recessive gene, relationships between genotypes and phenotypes, too. We irradiated the seed of sweet sorghum varieties by gamma-rays with optimum dosage (acute irradiation) or grew plants 10 - 30 m from 60Co source in the Gamma Field (chronic irradiation), Institute of Radiation Breeding, National Institute of Agrobiological Sciences. Survival rate of sorghum varieties by gamma-ray acute irradiation was different and differences of radiosensitivity to gamma ray in varieties are suggested. Various mutants were identified in the M2 population of these varieties. Spontaneous, naturally occurring, recessive mutations called bloomless (bm) and brown mid-rib (bmr) have been successfully utilized in sorghum breeding for forage use. These characteristics are also suggested to improve sorghum bioethanol production through fermentation of stems. Bloomless mutants and brown mid-rib mutants from three varieties were successfully obtained. The genetic analyses of the mutations were conducted by hybridization of the mutants with the original three varieties and one natural mutant demonstrated that these locus of bloomless character were different. Characterization of the mutations is being conducted by the use of genomic data. Artificial bmbmr plants were obtained by the hybridization of a bm mutant and a bmr mutant. Advantages of mutation induction by gamma-ray for genome analysis will be discussed.

# Phenotypic Assessment of Yield and Nutritive Values of Italian ryegrass (*Lolium multiflorum*) from a Spaced-Plant Field Trial

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Italian ryegrass (*Lolium multiflorum*) is an agronomically important pasture grass. Molecular breeding of Italian ryegrass for yield and quality through marker-assisted selection (MAS) has progressed little beyond the stage of QTL identification by trait-dissection. In order to effectively implement MAS for complex outcrossing species like Italian ryegrass, genomic selection strategies may be required. Genomic selection uses information on sequence variation across the whole genome to provide prediction equations for phenotypic performance for complex quantitative traits such, as yield. The objective of this study was to provide data for genome-wide association analysis and to identify superior genotypes for selection in breeding. A total of 960 Italian ryegrass genotypes were assessed for herbage yield and nutritive values in a field-based nursery experiment located at Hamilton in south-western Victoria, Australia, in 2010-2011. The experimental design was a randomised complete block with four replicates resulting in total 3840 plants in the trial. Herbage yields were recorded from 5 cuttings over the year. Nutritive values including crude protein, water-soluble carbohydrate, acid and neutral detergent fibre, and digestibility were assessed using near infra-red reflectance spectroscopy on vegetative samples harvested in May 2011. A broad range of phenotypic variation was observed. There were significant genotypic variance components for yield and nutritive values. Moderate heritability estimates were obtained for nutritive values, as compared to low to high heritability estimates for yields from different cuttings. Target plants were also genotyped using single nucleotide polymorphism (SNP) markers. While preserving all SNP allele variants, genotypes with high yield were selected for pair-crossing and evaluation of the derived families is being progressed. Phenotypic measurements from this study are amenable to genome-wide association studies, given provision of sufficient marker coverage over the genome, and investigation of the feasibility of genomic selection in Italian ryegrass breeding.

## Progress of the Pasture and Turf Breeding and Genetics Program of the Forage and Range Research Laboratory

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The breeding and genetics programs of the USDA ARS Forage and Range Research Laboratory include focus on cool-season grasses for pasture and turf production. The major objective of this program is the development of pasture grasses with high biomass production and nutritive value and turfgrasses with high turf quality that also possess high water-use efficiency and winterhardiness. Research efforts focus on evaluation of novel sources of germplasm, identification of genetic mechanisms controlling traits of interest, and development of elite breeding populations used for germplasm/cultivar release. Crop improvement is on-going in orchardgrass (cocksfoot) and timothy for pasture production and in Kentucky (meadow) bluegrass and rhizomatous wheatgrasses for turf production. Highlights of this research include the identification of potential heterotic groups for semi-hybrid orchardgrass development, the development of an EST library and linkage map for the autotetraploid orchardgrass genome, identification of Kentucky bluegrass accessions with salinity tolerance similar to that of tall fescue, and development of rhizomatous wheatgrass breeding lines with high turf quality and low irrigation requirements. Genetic objectives include elucidation of the role of water-soluble carbohydrate concentration on abiotic stress tolerance and identification of the genetic determinants of heading date. Research involves collaboration with public and private entities within North America and worldwide.



# Quantifying selfing and outcrossing in lowland Switchgrass populations using SSR markers

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Switchgrass (*Panicum virgatum* L.), a native C4 perennial species, is being developed as a major-cellulosic crop for biofuel production in the U.S. Previous reports indicated switchgrass plants set a few or no seed when their inflorescences were bagged. However, no information is available on mating behavior of plants under open pollinating conditions in the field. Accordingly, the objective of this study was to quantify selfing and outcrossing rates of switchgrass plants grown in the field. Two small (NL94 C2-3 and SL93 C2-3) and two large (NL94 C3 and SL 93 C3) lowland switchgrass breeding populations field established with three replications were used in the experiment. Each small population consisted of five parents, while each large population was composed of 26 parents. Half-sib (open-pollinated) seed samples in the four populations were hand collected from each parent plant on October 1, 2010. Ten seedlings per replication for each family were developed in a greenhouse on the Agronomy Research Station, OSU. DNA samples isolated from 1680 half-sib progeny with 168 female parents were genotyped with a set of six Simple Sequence Repeat (SSR) markers to estimate selfing versus outcrossing rates. Of the NL94 C3 offspring, 12 were selfed progenies (1.74%). While 18 half-sib families of NL C3 population did not contain any selfed progeny, progenies of eight families encompassed selfed individuals ranging from 3.3% to 13.3% on the basis of each family. In the population of NL94 C2-3, three were selfed progenies (2%). Interestingly, all progenies from SL93 C2-3 were derived from outcrossing. SSR marker analysis of SL93 C3 progeny plants is in progress. The findings would be valuable in the better understanding of reproduction systems and development of new cultivars in switchgrass.

## Relationships among orchardgrass subspecies

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*Dactylis* L. [orchardgrass or cocksfoot] is a monospecific genus used as a forage and hay grass. Nearly all cultivars of *Dactylis* are auto-tetraploid species *glomerata* ssp. *glomerata*, but there are also many other subspecies with diploid and tetraploid cytotypes. The diploid and tetraploid subspecies are maintained as accessions at seedbanks across the world, and many of the subspecies have not been characterized since initial taxonomic analyses at the time of collection. Additionally, these subspecies represent valuable secondary germplasm that have periodically been introgressed into orchardgrass variety development programs. Since categorization into subspecies has been inconsistent, it is possible that subspecies identifications would not always correspond to DNA-marker based groupings. In this study we have collected plants from 104 *Dactylis* accessions representing approximately 20 different subspecies/taxa, conducted nuclear DNA content analysis, and used SSR marker genotyping to estimate genetic relationships (or genetic diversity). Of the accessions, 35 were diploid, 56 tetraploid, and 13 were mixed diploid/tetraploid. Mean nuclear DNA content varied from 3.65 pg/2C (*D. masei*) to 4.97 pg/2C (*D. g.* subsp. *woronowii*) for diploid taxa, and from 8.18 pg/2C (*D. g.* subsp. *aschersoniana*) to 9.11 pg/2C (*D. g.* subsp. *woronowii*) in tetraploid taxa. No plants with hexaploid DNA content were detected. DNA markers highlighted subspecies with statistical support, while others were scattered among clades of varying subspecies. Implications of orchardgrass subspecies relationships will be discussed.

## Selection of *Paspalum* spp. accessions for use as turfgrass

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*Paspalum* spp. is a large genus with more than 400 identified species grouped into 20 taxonomic groups. In Brazil, there are around 220 native species. Embrapa Cattle-Southeast located in São Carlos, state of São Paulo, maintains a large germplasm bank of the genus with more than 320 accessions of 37 species collected in various regions of the country. Recently, a project coordinated by Embrapa Cattle-Southeast was initiated with the objectives of evaluating accessions for use as turf. An experiment was conducted in Campo Grande, MS, with 27 accessions belonging to ten species in plots 1.0 to 1.5 m<sup>2</sup> in three replications. Plots were established with individual cuttings spaced 15 cm from each other on February 2011 and evaluated for plant survival and soil cover. In the beginning of the rainy season in November, plots were cut to a 5 cm height and growth above that was harvested every fortnight in a 1 m<sup>2</sup> area. Data were analyzed by SAS. Of the 27 accessions, nine presented more than 80% survival 45 days after planting, and more than 80% soil cover 4 months after planting. These are: *P. modestum* 2 and 5, *P. lividum* 4, *P. rhodopedum* 8, *P. notatum* 13, 20 and 22 and *Paspalum* sp. 18. *Paspalum oteroi* 12 showed little plant survival but good soil cover. One year after planting soil cover varied from 3.4 m<sup>2</sup> to 32 m<sup>2</sup>. Total dry matter in the rainy season varied from 0.3 to 1.0 kg/m<sup>2</sup> in a maximum of 11 harvests. Accessions 2, 4 and 5 produced less than 400 g in 9 harvests. Considering highest soil cover and lower number of harvests, dry matter yields and flowering, accessions of *P. modestum*, *P. oteroi* and *P. lividum* showed great promise to be used as turf, emphasizing the great potential of this germplasm collection.

## The National BioResource Project *Lotus japonicus* and *Glycine max* / soja in Japan

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The National BioResource Project (NBRP) aim is to collect, preserve and provide bioresources that are basic materials for life sciences research, and to upgrade the bioresources responding to the demands of the present age by adding higher values through developing preservation technology, genome analysis, and others systematically. In addition, reinforcement of the function of the information center, which provides information of whereabouts and others, is included. Japanese trefoil (*Lotus japonicus*) is a wild perennial plant with a small genome and a short life cycle. This plant is expected to play a role as the model organism of leguminous plants, which include important crop plants. Additionally, the soybean, *Glycine max* (L.) Merr., is the most important grain legume crop in terms of total production and international trade of agricultural products. Legume Base, a resource center for *L. japonicus* and *G. max*, was established in April 2004. The scope of Legume Base is the collection, development and conservation of the genetic resources of *L. japonicus* and *G. max* and the distribution for the utilization by the research community. DNA resources including genomic DNA clones will be also available through Legume Base web site (<http://www.legumebase.brc.miyazaki-u.ac.jp>). In here, we will introduce NBRP Japan and Legume Base.

# Biosynthesis of Proanthocyanidins in White Clover Flowers: Cross Talk within the Flavonoid Pathway

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Proanthocyanidins and anthocyanins are produced by closely related branches of the flavonoid pathway and utilize the same metabolic intermediates. Previous studies have shown a flexible mechanism of flux diversion at the branch-point between the anthocyanin and proanthocyanidin pathways, but the molecular basis for this mechanism is poorly understood. Floral tissues in white clover (*Trifolium repens*) plants produce both proanthocyanidins and anthocyanins. This makes white clover amenable to studies of proanthocyanidin and anthocyanin biosynthesis and possible interactions within the flavonoid pathway. Results of this study show that the anthocyanin and proanthocyanidin pathways are spatially co-localized within epidermal cells of petals and temporally overlap in partially open flowers. A correlation between spatio-temporal patterns of anthocyanin and proanthocyanidin biosynthesis with expression profiles of putative flavonoid-related genes indicates that these pathways may recruit different isoforms of flavonoid biosynthetic enzymes. Furthermore, in transgenic white clover plants with down-regulated expression of the anthocyanidin reductase gene, levels of flavan 3-ols, anthocyanins, and flavonol glycosides and the expression levels of a range of genes encoding putative flavonoid biosynthetic enzymes and transcription factors were altered. This is consistent with the hypothesis that flux through the flavonoid pathway may be at least partially regulated by the availability of intermediates.

## Characterization of *Medicago truncatula* mutants and application of the knowledge for alfalfa improvement

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*Medicago truncatula* has been developed into a model legume. Many tools and resources have been developed in *M. truncatula*. By screening a large population of Tnt1 retrotransposon-tagged mutants of *M. truncatula*, we identified several types of mutants that exhibited the defects in compound leaf development, leaf senescence and seed development. To apply the information gained from *M. truncatula* to improve hay quality of alfalfa, we focused on a leaf senescence related mutant that maintained green leaves during senescence. Genetic and molecular analyses revealed that the mutation was caused by Tnt1 insertion in a *M. truncatula* STAY-GREEN (MtSGR) gene, which is induced by senescence and responsible for chlorophyll breakdown. Transcript profiling revealed that large numbers of genes were either up-regulated or down-regulated in the mutant. Based on the MtSGR sequence, an alfalfa STAY-GREEN gene (MsSGR) was cloned, and transgenic alfalfa lines were produced by RNA interference of MsSGR. Silencing of MsSGR led to the production of stay-green transgenic alfalfa. This beneficial trait offers the opportunity to produce premium alfalfa hay with a more greenish appearance. Furthermore, most of the transgenic alfalfa lines retained more than 50% of chlorophylls during senescence and had increased crude protein content. This study illustrates the effective application of the knowledge from a model system for the genetic improvement of an important commercial crop.

# Elucidating Condensed Tannin Storage in the Leaves of Legume Species

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Condensed Tannins (CT) are one of several classes of phenylpropanoids synthesized and accumulated by many plant species including legumes. Condensed tannins are synthesised by the phenylpropanoid pathway, involving an array of structural and regulatory genes for the production of monomeric precursor units, polymerisation of these precursors and eventual transportation into the vacuole for storage. Unlike the biosynthesis and sequestration of anthocyanins, the equivalent mechanism for CT accumulation in plant cells is poorly understood. We needed to gain an insight into positioning and storage of CT in legume species. We used various microscopic and staining techniques to establish the cellular position of CT within leaf tissue of a range of non-forage *Trifolium* species and other forage legumes known to accumulate significant CT levels. There was a continuum of CT storage patterns within the leaves of legume species. White clover leaf CT were restricted to the abaxial trichomes and synthesized exclusively during meristematic development prior to leaf emergence. In *T. arvense*, leaf CT were located along the inner plant epidermis in most abaxial and adaxial leaf cells but did not fill the vacuolar space. In contrast *Lotus corniculatus*, *L. pedunculatus* and *Desmodium uncinatum* exhibited a widespread storage pattern, with CT located throughout the leaf mesophyll and palisade cells, but absent in any epidermal cells. These results indicate that different storage patterns exist in related species and are probably governed by differential regulation. Utilization of this information has implications for functional analysis and manipulation of the CT pathway.

# High-throughput Automated Low-Cost Quantification of Individual Water Soluble Carbohydrates and Protein in Grass Hbage

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Quantification of forage quality is essential for the identification of elite genotypes and the continued advancement of pasture grass breeding. A number of methods for quantification of water soluble carbohydrates (WSC) and plant protein are available, such as near-infra red spectroscopy (NIRS) and high performance liquid chromatography (HPLC). However, such methods are labor intensive, low-throughput and cost-prohibitive for commercial breeding programs, which typically need to assess thousands of samples annually. An accurate high-throughput micro-plate-based protocol has been developed and validated, with the ability to simultaneously process and quantify WSC and plant protein with a high level of automation. This protocol represents an important throughput improvement in pasture plant phenotyping, with an increase in sample processing of c. 11-fold compared to commonly-used methods. As WSC and protein are extracted simultaneously and quantified within micro-plates, consumable costs are minimized with optimal reagent use efficiency, resulting in a low cost per sample that is suitable for commercial pasture breeding companies. This represents a first high-throughput, low cost herbage quality phenotyping protocol suitable for broad-scale application which allows breeders to select elite genotypes based not only on visual assessment but also on WSC-to-protein ratios for improved ruminant nutrition.

# Lignin Biosynthesis in *Paspalum dilatatum*: Isolation and Characterisation of Cinnamoyl CoA Reductase

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Dallisgrass (*Paspalum dilatatum* Poir) is a highly productive C<sub>4</sub> grass native to South America and naturalized in Australia with special relevance for dairy and animal production. However, digestibility of warm-season grasses is poor compared with most temperate grasses. The main factor limiting digestibility is lignin deposition in plant cell walls which causes resistance to digestion by ruminant microbes. During the transition of the vegetative to the reproductive stage of development, lignin composition in monocots changes with an increase in syringyl (S) lignin and decrease in guaiacyl (G) lignin that negatively correlates with digestibility. The aim of this work is the identification of genes associated with lignin biosynthesis and the isolation and characterisation of cinnamoyl CoA reductase (CCR) gene in *P. dilatatum*. Lignification-related candidate genes were identified by transcriptome sequencing using the Roche GS FLX sequencing platform. A novel PdCCR with a coding sequence of 1,293 bp from *P. dilatatum* was isolated. Phylogenetic analysis shows that PdCCR is closely related to other monocotyledonous CCRs revealing with high homology to SbCCR. Deduced amino acid sequence analysis showed that the catalytic site for CCR enzymatic activity and the conserved NAD/NADP(H)-dependent dehydrogenase and reductase binding fold domains were present in PdCCR. The genomic analysis revealed that CCR belong to a multicopy gene family in *P. dilatatum*. The spatio-temporal profile of lignin deposition shows an increase in S lignin and G lignin deposition in cell walls and the accumulation of cells enriched in S lignin during plant maturity. The expression profile of PdCCR correlates with plant maturation and lignin deposition. This work describes the characterisation of a CCR gene in *P. dilatatum* for the first time contributing to the knowledge base of the functional characterisation of lignin related genes in warm-season grasses.

## Cumulative potential net benefits of transgenic white clover and alfalfa for dairy production in southern Australia

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White clover (*Trifolium repens* L.) and alfalfa (*Medicago sativa* L.) are key legume forages used for grazing and fodder conservation by the southern Australian dairy industry. White clover and alfalfa with alfalfa mosaic virus resistance ('AMV Res.') and delayed leaf senescence ('DLS') have been developed and field evaluated (G. Spangenberg, pers. com.). As these forages are yet to be tested under commercial farming conditions, we have developed an economic model to estimate the potential net benefit of these species and technologies under Australian farming conditions. For a mixed pasture sward containing white clover and perennial ryegrass (*Lolium perenne* L.), the cumulative net benefits were estimated over 10 years while for the alfalfa hay stand a four to five year period was used. Partial budget analysis was used to determine the potential value of including the 'AMV Res.' and 'DLS' traits individually, and in combination as a trait stack. This approach compared the annual potential extra benefits minus the annual potential extra costs from growing the novel forages compared to a current common cultivar. Based on the 'what if' assumptions used in this analysis the traits showed the greatest median potential net benefit, and greatest variability of potential net benefit, when deployed in combination compared to single traits under all scenarios tested. Sensitivity analysis was used to identify the key contributing factors to potential value creation under all scenarios. The use of economic models accompanying molecular breeding programs can inform the prioritisation of traits deployed and also assist in determining required trait efficiencies.

# In vitro Chemical Mutagenesis of Apomictic Bahiagrass for Improvement of Turf Quality

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Tetraploid apomictic bahiagrass (*Paspalum notatum* Flugge) cultivar “Argentine” is a prime low-input and drought tolerant turf and forage species. However, the turf quality of bahiagrass is limited by its open growth habit, light green color and prolific production of tall seedheads. Genetic improvement by conventional breeding is very difficult due to its apomictic mode of reproduction. Our objective was to explore the potential of in vitro chemical mutagenesis for generation of uniform mutagenized seed progeny with improved turf quality. Scarified and surface sterilized bahiagrass seeds were treated with different concentrations of the mutagen sodium azide. Callus was induced from these seeds and regenerated via somatic embryogenesis to obtain uniformly mutagenized plants. 2,000 of the 20,000 regenerated seedlings were selected based on their morphological characteristics and transferred to soil. 46 independently mutagenized lines (M1 lines) with reduced stem length, higher tiller density or reduced or delayed seedhead formation were established under field conditions in 1.2m x 1.2m plots in a randomized block design with 4 replications for further evaluation of density, leaf texture, tiller length, color, growth pattern, biomass, seedhead and seed production, as well as seedling vigor. Mutagenized lines with improved characteristics and production of viable seed were identified and their apomictic M2 progeny was evaluated in 3m x 3m plots. The superior line displayed higher density, finer leaves, an upright growth habit, dark green color, reduced seedhead formation and uniform seed progeny. This line also retained the superior drought tolerance and persistence of bahiagrass.

## Analysis of Compatibility and Stability in Designer Endophyte-Grass Associations between Perennial Ryegrass and *Neotyphodium* Species

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Fungal species of the genus *Neotyphodium* form endophytic symbioses with agronomically important pasture grass species such as perennial ryegrass (*Lolium perenne* L.) and tall fescue (*Festuca arundinacea* Schreb.). The fungal mycelium proliferates within the vascular tissue of aerial tissues, especially the leaf base and leaf sheath, and is asexually propagated through colonisation of seeds. Fungal grass endophytes produce both beneficial (tolerance to abiotic stresses such as drought, deterrence of invertebrate herbivores) and deleterious effects (toxicity to mammalian herbivores) for pasture grass production. Herbivore toxicity effects are associated with production of alkaloids such as lolitrem B and ergovaline (producing mammalian toxicity syndromes such as ryegrass staggers and fescue toxicosis) and peramine (deterrence of feeding by insect pests such as the Argentine stem weevil). A genetic diversity study based on gene-associated simple sequence repeat (SSR) markers has identified the range of limited but significant global variation within *N. lolii*. This variation can be correlated with both geographical origin in Eurasia and major toxin profiles. Using genotypic data as a predictor of likely toxin profile, a germplasm collection resource established on the basis of geographical targeting and genotypic analysis identified a discrete number of previously unidentified endophyte strains, some of which also have favourable toxin profiles. These novel endophytes have been deployed into a novel method for inoculation of multiple strains into common genetic backgrounds of host grass, providing a panel of symbiota suitable for isogenic comparisons based on identity of either the grass or endophyte genotype. Members of the isogenic inoculation panel have been used to assess compatibility and intergenerational stability of the symbiota dissecting endophyte and host plant genotype effects.

# Analysis of Compatibility and Stability in Designer Endophyte-Grass Associations between Tall Fescue and *Neotyphodium coenophialum*

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*Neotyphodium* fungal species form endophytic symbioses with agronomically important pasture grasses. Tall fescue (*Lolium arundinaceum* [Schreb.] Darbysh syn. *Festuca arundinacea* Schreb.) is generally reported to associate with the endophyte *Neotyphodium coenophialum*. Both beneficial and detrimental agronomic properties result from the association, including improved tolerance to water and nutrient stress and resistance to invertebrate pests. Invertebrate resistance is provided by specific metabolites produced by the endophyte, in particular loline alkaloids and peramine. Other metabolites produced by the endophyte, such as ergot alkaloids, are toxic to grazing animals and reduce herbivore feeding. Genetically novel endophyte strains with favourable alkaloid profiles have been identified in a study of global genetic diversity using expressed sequence tags (EST)-derived simple sequence repeats (SSR) markers combined with metabolic profiling. Novel endophytes have been isolated for inoculation into an isogenic host plant genotype panel selected from elite tall fescue germplasm to assess endophyte compatibility, and vegetative stability, with the host plant genotype. Nine out of 10 novel endophytes strains were successfully inoculated into four host genotypes representing a broad range of tall fescue cultivars. Following inoculation, in-depth characterisation of the designer symbiota (i.e. endophyte-host associations) can be performed independent of effects due to host genotype variation.

## Comparison of alkaloid gene clusters in three endophyte isolates from drunken horse grass

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Drunken horse grass (*Achnatherum inebrians* (Hence) Keng) is a competitive perennial intoxicating grass, growing on alpine and subalpine grasslands of Northwest China, and is associated with grassland degradation. Livestock tend to avoid *A. inebrians*, which is known to be toxic to cattle, sheep, goats and horses. Endophytes of this grass play an important role in both competitiveness and toxicity, which is attributable to their production of alkaloids that accumulate to high levels in the plant. Two different endophyte taxa are reported in *A. inebrians*: *Neotyphodium gansuense* Li et Nan (Ng) and *N. gansuense* var. *inebrians* C. D. Moon et Schardl (Ngi). We sequenced the genomes of an Ng isolate from Northwest Gansu Province and two Ngi isolates from Southeast Gansu Province and Xinjiang Province, China. We identified an ergot alkaloid biosynthesis (EAS) gene cluster but no indole-diterpene biosynthesis (IDT) gene cluster in the two Ngi isolates. Plants with these isolates had high levels of the ergot alkaloids, ergonovine and lysergic acid amide. In contrast, the Ng isolate had an IDT cluster but no EAS cluster. We inoculated endophyte-free (E-) *A. inebrians* with Ng, and the infected plants (E+) are ready to check for indole-diterpenes. The diversity of endophytes in drunken horse grass with their different alkaloid profiles may have important implications for management of grazing livestock on the grasslands of Northwest China.

# De Novo Generation of Genetic Diversity in *Neotyphodium* Grass Fungal Endophytes Based on Colchicine Treatment

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Colchicine inhibits chromosome segregation during mitosis and has been widely used for induction of chromosome doubling in plants, but limited to very few fungal species. This study has developed a method for putative induced polyploidisation in endophytic symbiotic fungi of perennial ryegrass (*Lolium perenne* L.). The *Neotyphodium* endophyte strain PT (non-*N. lolii* taxonomic group) was grown in potato dextrose broth in the presence of 0.1-0.2% (w/v) colchicine. Protoplasts were prepared from colchicine-treated (CT) mycelia and then used to regenerate single fungal colonies. Over one hundred CT colonies were regenerated from protoplasts, of which 20 were randomly selected for further analysis. SYBR Green staining of nuclei and flow cytometric analyses indicated that the majority (80%) of CT strains contained similar DNA content to the PT (parental) endophyte. Four CT strains exhibited an increase in nuclear DNA content, although whole genome duplication was not observed. In vitro growth assays showed that, when compared to PT endophyte, one strain exhibited enhanced growth, while two CT colonies showed reduced growth. Four of the 20 CT colonies showed increased antifungal activity against several plant fungal pathogens. The genomes of 10 CT strains were sequenced using the HiSeq 2000 Sequencing System. No large partial duplications events were detected in any of the strains. However, genome assembly produced contigs that were greater in abundance but smaller in size compared to the assembled PT genome. It is speculated that the increased abundance of smaller contigs in CT strains may be caused by increased transposon prevalence. Differences in genome assembly statistics indicate that genomic changes caused by colchicine treatment have occurred in CT strains. The use of colchicine may hence be applicable to the de novo generation of genetic diversity in *Neotyphodium* endophytes including the production of artificial polyploid endophytes exhibiting novel traits such as enhanced growth and host colonization and increased antifungal activity.

# De Novo Generation of Genetic Diversity in *Neotyphodium* Grass Fungal Endophytes Based on X-Ray Mutagenesis

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The fungal endophyte *Neotyphodium lolii* is a common symbiont of perennial ryegrass (*Lolium perenne* L.). In this study, a method for generating novel endophyte genetic diversity using ionising radiation is described. Liquid cultures of *N. lolii* were exposed to a single or double dose of ionising radiation (from the radioisotope Cesium-137) ranging from 10–30 Gray (Gy). After a period of recovery, protoplasts were prepared from irradiated mycelia and then used to regenerate single fungal colonies. More than 5,000 irradiation-mutagenised (IRM) colonies were regenerated from protoplasts. Nine IRM colonies, which were subjected to different doses of radiation, were selected for further analysis. All 9 IRM colonies exhibited reduced in vitro growth compared to the non-irradiated standard toxic (ST) endophyte. However, in a dual-culture in vitro assay, these IRM colonies showed similar activity against several fungal pathogens as the ST endophyte. The genomes of the 9 IRM colonies were sequenced using the HiSeq 2000 Sequencing System. Sequence analysis revealed that three IRM strains contained large (>250 bp) deletions, including those within genes encoding proteins of predicted function. Three other IRM strains contained partial duplications of chromosomal regions. Compared to the ST endophyte reference genome, all IRM strains contained approximately 3-5 single nucleotide polymorphisms (SNPs) and 100 small insertions/deletions (INDELs) per Mb across genic regions. The effect of radiation dose on the mutagenesis index was also compared among the IRM strains. A repeated dose of 10 Gy on endophyte mycelia created the highest number of SNPs and INDELs per Mb across genic regions. Use of ionising radiation consequently has the potential for de novo generation of genetic variation in *Neotyphodium* endophytes.



# Genetic Diversity and Host Specificity of Fungal Endophyte Taxa in Fescue Pasture Grasses

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A number of pasture and turf grass species form mutually beneficial symbiotic associations with endophytic fungal species. Within the fescue grasses, diploid meadow fescue interacts with *Neotyphodium uncinatum*, while allohexaploid tall fescue has been reported to associate with *N. coenophialum* and two other morphologically distinct taxa (FaTG-2 and FaTG-3). The evolutionary history of hexaploid tall fescue is complex, as it is part of a species group with varying ploidy levels, and exhibits distinct ecogeographical morphotypes. In order to evaluate both naturally occurring variation and host grass taxon specificity, diversity was determined in collections representing multiple meadow fescue and tall fescue accessions. Initial screening with a minimal set of endophyte-specific SSR genetic markers detected endophyte incidence in 33% of 701 tested accessions. Subsequent analysis identified *N. coenophialum* genotypes within Continental and rhizomatous hexaploid and octoploid tall fescue accessions. FaTG-2 and FaTG-3 endophytes appeared to be restricted to Mediterranean hexaploid and decaploid tall fescue hosts. Endophytes of meadow fescue were confirmed as belonging to *N. uncinatum*. This study has elucidated host specificity of fescue endophyte taxa and supported models for host-symbiont co-evolution.

## Metabolic Profiling of Novel *Neotyphodium* Endophytes in Tall Fescue (*Lolium arundinaceum* Schreb.)

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Tall Fescue (*Lolium arundinaceum* [Schreb.] Darbysh syn. *Festuca arundinacea* Schreb.), which is one of the most economically important forage grasses in temperate regions of the world, is known to form associations with *Neotyphodium* species endophytes, of which *Neotyphodium coenophialum* is the most highly characterised taxon. In addition, endophytes belong to other groupings such as FaTG-2, FaTG-3 (*Festuca arundinacea* Taxonomic Group) and other uncharacterised taxa have also been identified to reside within tall fescue. Endophyte symbiosis confers protection from mammalian and insect herbivory through the production of a range of secondary metabolites in planta. Among them, peramine, ergot alkaloids, lolines and lolitrems provide protection to the host plant from insects while lolitrems and ergot alkaloids are also toxic to grazing animals. Knowledge of the alkaloid composition of the symbiotum metabolome is consequently an essential component for selection of agronomically favourable endophytes which do not produce mammalian toxins, but still synthesise beneficial alkaloids for resistance to invertebrate herbivory as well as ecological fitness. Twenty novel tall fescue endophyte strains from five taxa were characterised for in planta production of the above four alkaloids using liquid chromatography-mass spectrometry (LC-MS). The results revealed diverse alkaloid profiles between strains and endophyte taxa. Six endophyte strains were identified as lacking production of both ergovaline and lolitrem B. Endophytes belonging to the presently unclassified 'non-Epichloë outgroup' failed to produce any of the known alkaloids. Novel endophytes with favourable alkaloid profiles have been isolated and inoculated into an isogenic host plant genotype panel in order to perform detailed characterisation of the metabolic profiles of novel endophytes independent of effects due to host genotype variation.

# Pan-Genome Analysis of Perennial Ryegrass Endophytes

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*Neotyphodium* species are fungal endophytes that form symbioses with agronomically important pasture grasses. *N. lolii* is the predominant endophyte of perennial ryegrass (*Lolium perenne* L.), but other non-*N. lolii* taxa, such as LpTG-2, are also detected. Within- and between-taxon diversity provides a source of variation for production of secondary metabolites (in particular lolitrem B, ergot alkaloids and peramine) which are either toxic to grazing animals or reduce feeding by invertebrate herbivores. A selection of 23 perennial ryegrass endophytes was assembled in order to sample diversity across the range of variation for the various taxa, and high-throughput DNA sequencing technology has been performed on each strain. Furthermore, the genome of the commonly identified 'Standard Toxic' (ST) *N. lolii* endophyte has been assembled into a reference genome, in order to facilitate this cross-taxon pan-genome analysis. Results from the analysis include: an enhanced understanding of the genomic variation amongst various perennial ryegrass endophytes; improved insights into mitochondrial and nuclear genomic changes that have occurred during the endophyte evolution; and an increased ability to associate genetic variation with phenotypic differences, such as presence and absence of key genes in alkaloid biosynthesis.

## Systems biology of alkaloid biosynthesis in fungal endophytes of tall fescue (*Lolium arundinaceum* Schreb.)

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Symbiotic associations between tall fescue grasses and endophytic fungi of *Neotyphodium* species manifest enhanced resistance to several biotic stress factors, including protection from mammalian and insect herbivory and resistance to both nematode pests and some fungal pathogens. The ability of endophytes to synthesise a range of secondary metabolites in planta plays a major role in obtaining protection from mammalian and insect herbivores. Grasses infected with endophytes predominantly produce four varieties of alkaloids: pyrrolopyrazine alkaloids (e.g. peramine); ergot alkaloids (e.g. ergovaline); pyrrolizidine (e.g. lolines); and indole diterpenes (lolitrems). However, different endophyte strains have diverse alkaloid production capabilities. In this systems biology (genome-metabolome) study, whole genome sequencing of several novel tall fescue endophyte strains that exhibit phenotypic differences, including diverse alkaloid production profiles, was performed using the HiSeq2000 (Illumina) DNA sequencing platform, to identify sequence polymorphisms. For instance, the content of genes that involved in alkaloid biosynthesis was compared between novel endophyte strains, including the taxa *N. coenophialum*, FaTG-2, FaTG-3 (*Festuca arundinacea* Taxonomic Group) and a currently uncharacterised 'non-Epichloë out-group'. Deletions were observed in regions harbouring genes associated with alkaloid production that correlated with variation for known alkaloid profiles. Further analysis is ongoing to relate other phenotypic differences observed among these endophyte strains and their sequence variation.

# Understanding alkaloid diversity in tall fescue endophytes

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Epichloid endophytes (*Epichloë* and *Neotyphodium* sp.) associate with cool-season grasses, including the agriculturally important forages tall fescue and perennial ryegrass. This association confers protection from a variety of biotic and abiotic stresses, including herbivory and drought. A collection of 85 tall fescue lines from 15 locations in Greece, including both Continental (summer active) and Mediterranean (summer dormant) germplasm, was screened for the presence of native endophytes. A total of 37 endophyte-infected lines from 10 locations were identified and the endophytes were classified into five distinct groups based on physical characteristics and molecular markers designed to alkaloid biosynthesis genes and SSR sequences. These classifications were supported by phylogenetic analyses of the housekeeping genes *tefA* and *tubB*, and the endophytes were further categorized as *N. coenophialum* (represented by three independent groups) or *Neotyphodium* sp. FaTG-2 (represented by two independent groups) isolates. Analyses of the tall fescue *matK* chloroplast genes indicated a population-wide, host-specific association between *N. coenophialum* and Continental tall fescue and FaTG-2 with Mediterranean tall fescue, which also correlated with differences in colonization of host tillers by the native endophytes. The alkaloid potential of the endophytes was determined using a PCR-based gene profiling approach of infected plant material for the presence or absence of all genes known to be required for the production of each alkaloid class. All alkaloid potential predictions, except for one, were validated by chemical analyses of infected plant material. Except for the gene responsible for the production of peramine in one of the FaTG-2 groups, variation in alkaloid gene content, specifically the presence and absence of genes, and copy number of gene clusters explained the chemotype (chemical phenotype) diversity observed in the tall fescue collection. The results from this study provide insight into endophyte germplasm and diversity present in tall fescue collections.

# Assessment of Gene Flow in White Clover (*Trifolium repens* L.) under Field Conditions in Australia Using Phenotypic and Genetic Markers

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White clover is one of the most important pasture legumes in global temperate regions. It is an outcrossing, insect-pollinated species with gene flow occurring naturally between plants. A 2-year study was conducted to assess the relationship between gene flow and physical distance in white clover under field conditions in southern Australia. White clover plants exhibiting a red leaf mark phenotypic trait acted as pollen donors to recipient plants lacking leaf markings at distances up to 200 m distant from the donor plants. Progeny were scored for the dominant red-leaved phenotype and gene flow was modelled. Paternity was confirmed using simple sequence repeat markers. A leptokurtic pattern of gene flow was observed under conditions designed to measure maximised gene flow with the majority of pollination occurring in the first 50m from the donor pollen source.

# Development of an Antibiotic Marker-Free Creeping Bentgrass with Resistance to two Herbicides

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Creeping bentgrass (*Agrostis stolonifera* L.) is one of the most widely used turf species in golf greens around the world. When a golf green is established, weeds hinder early growth and settlement of the grass, decreasing productivity in the green, and interfering with its later growth. To prevent the emergence of herbicide-resistant weeds when only one herbicide is in use, we sought to develop a new dual-resistance creeping bentgrass to facilitate mixture processing with much lower density or systematic approach through a combination of non-selective herbicides. Herbicide-resistant creeping bentgrass without antibiotic resistance markers were produced through Agrobacterium-mediated genetic transformation method. Embryogenic callus were infected with Agrobacterium tumefaciens EHA105 harboring the bar and the CP4-EPSPS genes for bialaphos and glyphosate resistance, respectively. Phosphinothricin-resistant calli and plants were selected. Soil-grown plants were obtained 14-16 weeks after transformation. Stable transformation of the selected regenerated plants was validated by PCR and Southern blot analysis revealed that at least one copy of transgene was integrated into the genome of each transgenic plants. Transgene expression was further confirmed by northern blot analysis and the CP4-EPSPS protein was detected by ELISA. Transgenic plants remained green and healthy when sprayed with Basta containing 0.5% glufosinate ammonium or glyphosate. Agrobacterium-mediated transformation efficiency was high (9.4%). For the first time herein, we report the Agrobacterium-mediated transformation of bentgrass with two herbicide-resistance genes. Thus, this effective and reliable method could be used as routine transformation and may facilitate the development of new varieties of creeping bentgrass.

## Function Analysis of MwLEA3 gene of Mongolian Wheatgrass

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Mongolian wheatgrass (*Agropyron mongolicum* Keng), is a persistent, long-lived perennial grass. It not only has high feeding value, but also is rich in stress resistance genes, such as drought-tolerance, cold-tolerance, salt-tolerance and so on. It could provide valuable resource for the improvement of resistance and breeding of forage and its closely related species. In this study, the function of the MwLEA3 gene of Mongolian Wheatgrass was verified on the basis of molecular biological identification and physiological and biochemical identification. The plant expression vector of pCAM-MwLEA3 was constructed and transformed into tobacco via Agrobacterium infestation. T0 transformed tobaccos were tested by PCR, PCR-Southern, RT-PCR and imposition of drought stress. Molecular detecting results showed the MwLEA3 gene had been transformed into tobacco and expressed. The results of drought stress showed that the transgenic tobaccos had stronger resistance than wide-type ones. Moreover, we constructed the fusion protein vector pA7-MwLEA3 for subcellular localization, and it was transformed into onion epidermal cells, the results showed the expressed protein encoded by MwLEA3 was a nuclear localized protein.

# Generation of transgenic tall fescue overexpressing molecular chaperones for enhanced tolerance against abiotic stresses

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Forages are the backbone of sustainable agriculture and contribute extensively to the world economy. The majority of forage species have a complicated genome which makes conventional breeding difficult and painfully slow. Genetic transformation has become a powerful tool of molecular breeding for improving forage quality as well as tolerance to various abiotic stresses. Environmental stresses, such as drought, salinity and extreme temperatures significantly increase the generation of reactive oxygen species (ROS). ROS interrupts important cellular processes resulting in a significant reduction of plant growth. Therefore, exploitation of the mechanisms that protect plant from ROS is a major target to engineer forage crops for stress tolerance. The 2-cysteine peroxyredoxins exhibit both peroxidase and chaperon function in plants under stress condition. We overexpressed 2-cysteine peroxyredoxins under the control of a constitutive promoter in tall fescue. The transgenic plants showed significantly lower electrolyte leakage and lipid peroxidation under heat (42°C) or methyl viologen stress. Under heat stress, transgenic plants maintained their chlorophyll fluorescence (Fv/Fm) for more than 24hrs, while wild-types lost chlorophyll fluorescence very quickly. We hypothesize that the high levels of 2-cysteine peroxyredoxins proteins help transgenic plants to be protected from oxidative damage through its chaperon activity. The chloroplast is the major site of ROS generation in plants. Oshsp26 is a chloroplast-localized small heat shock protein that has been shown to express following oxidative or heat stress. Overexpression of the 'local' chaperone could play special protective role. Thus, we overexpressed Oshsp26 in tall fescue, which resulted in higher photochemical efficiency of PSII (Fv/Fm) in transgenic plants than that in wild types during heat stress. Our results suggest that overexpression of molecular chaperones increases cellular protection and enhance plant performance under abiotic stresses. (This work was supported by a grant from the Next-Generation BioGreen 21 Program (No. PJ008139), Rural Development Administration, Republic of Korea).

# Study on Transformation of AtCBF1 Gene mediated by *Agrobacterium Tumefaciens* in Alfalfa

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The purpose of the paper is improving alfalfa cold resistance effect by cold-induced transcription activator CBF1 gene. The AtCBF1 gene was amplified and cloned by PCR from genomic DNA of *Arabidopsis thaliana*. The resulting sequence demonstrated that the length of DNA cloned fragment is 642bp and the sequence homology rate was 99.84%, compared to the CBF1 gene sequence in GenBank by DANMAN sequencing. On this basis, the plant expression vector contained pBI121-CBF1 with AtCBF1 gene construct, and then the target gene was transformed into alfalfa using *Agrobacterium tumefaciens* methods. The transformed plants were selected by kanamycin. Furthermore, the target gene was detected by PCR and RT-PCR, and a 650bp band was demonstrated in the electrophoretic profile, which confirmed that AtCBF1 gene had been expressed in alfalfa. In summary, the research established the good basis of breeding new alfalfa varieties on cold resistance.

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